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(54) Title: COMPOSITIONS AND METHODS FOR THE TREATMENT OF SEVERE ACUTE RESPIRATORY SYNDROME (SARS)

(57) Abstract: The present invention provides compositions and methods for treating a coronavirus infection, especially a SARS CoV infection, are presented. The compositions comprise an antiviral nucleoside or mimetic thereof, or an antiviral antisense agent, in a form suitable for pulmonary or nasal delivery. The methods comprise administering to a patient in need thereof an antivirally effective amount of a composition according to the present invention, either by pulmonary or nasal instillation.



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## COMPOSITIONS AND METHODS FOR THE TREATMENT OF SEVERE ACUTE RESPIRATORY SYNDROME (SARS)

### FIELD OF THE INVENTION

5           The present invention is directed, in part, to compositions and methods for treating viral infections, such as coronavirus infections, by pulmonary or nasal administration of antiviral compounds and compositions comprising the same.

### BACKGROUND OF THE INVENTION

10           Coronaviruses have been known for some time. The first coronavirus isolated, in 1937, was the avian infectious bronchitis virus, which can cause devastating disease in chicken flocks. Since then researchers have found its cousins to infect cattle, pigs, horses, turkeys, cats, dogs, rats, and mice. Coronaviruses are grouped generally into three groups, two mammalian and one avian. Human coronaviruses (HCoV) are found in both group 1 (HCoV-229E) and group 2  
15 (HCoV-OC43) and are responsible for about 30% of mild upper respiratory tract illnesses. The first human family member was cultivated from nasal cavities in the 1960s, after researchers realized that another group, the rhinoviruses, was responsible for only about half of all common cold infections. Today, the two known human coronaviruses, OC43 and 229E, are thought to cause about 30% of cases, depending on the year. Small changes in the S glycoprotein can result  
20 in radical changes in a coronavirus' virulence and route of infection.

          Coronaviruses are large, enveloped, plus-stranded RNA viruses. They cause the common cold in all age groups accounting for approximately 15% of all colds. Coronaviruses have been implicated in the etiology of gastrointestinal disease in infants. They also cause economically important diseases in animals (e.g. avian infectious bronchitis and porcine  
25 transmissible gastroenteritis). Coronaviruses get their name because in electron micrographs the envelope glycoproteins appear to form a halo or corona around the periphery of the virion. The coronaviruses are also interesting because they are the only plus-strand RNA viruses with a helical nucleocapsid.

          There are two genera in the Coronaviridae family, Coronavirus and Torovirus. All  
30 human pathogens are in the Coronavirus genus. Prior to discovery of SARS-CoV, there were two identified groups of human coronaviruses, those resembling strains 229E and OC43, respectively. There is considerable antigenic heterogeneity, however, in all identified both

groups. For example, patients are found to make antibodies specific for both strains after a single infection.

The coronavirus genome is plus-stranded RNA 27 kb to 33 kb in length depending on the particular virus. The virion RNA has a cap at the 5' end and a poly A tail at the 3' end. The length of the RNA makes coronaviruses the largest of the RNA virus genomes. Coronavirus RNAs encode: 1) an RNA-dependent RNA polymerase; 2) N-protein; 3) the three envelope glycoproteins: E1, E2 and the hemagglutinin (HA); and 4) three non-structural proteins located between S and E, between M, and N, or downstream of N. These non-structural proteins, which vary widely among the different coronavirus species, are of unknown function and are dispensable for virus replication. The genome of the SARS-CoV (Urbani strain) is fully disclosed and discussed by Rota et al., Science, 2003, 300, 1394-9.

The genomes of coronaviruses usually contain more than 30,000 nucleotides, and they have a complex two-step replication mechanism. Coronaviruses can have up to 10 separate genes. Most ribosomes translate the longest gene, called replicase. The replicase gene produces a number of enzymes that use the rest of the genome as a template to produce a set of smaller, overlapping messenger RNA molecules, which are then translated into the so-called structural proteins--the building blocks of new viral particles. It is believed that interfering with the transcription or translation of the replicase gene in SARS-CoV will lead to inactivation of the replication cycle, thereby defeating the viral infection directly, delaying the onset of disease until the body can mount its own antiviral defenses, or some combination of these two effects.

Electron micrographs show that coronaviruses are pleomorphic in structure. Most virions appear roughly spherical with diameters in the range of 80-220 nm. The virion consists of a helical nucleocapsid surrounded by a membrane envelope. In the nucleocapsid, the single-stranded, non-segmented RNA genome is coated with N-protein. The virus membrane, which is derived from the host cell, contains three virus-encoded glycoproteins, E1, E2 and HA.

Human coronaviruses grow poorly in culture and cannot be analyzed in detail. Most studies are carried out with mouse hepatitis virus, a coronavirus that grows well in culture and is related to human strain OC43. Infection begins when the virus recognizes a cell surface receptor found to be aminopeptidase N for strain 229E and sialic acid for OC43. The virus enters the cell by endocytosis and membrane fusion.

As with most RNA viruses, coronavirus replication takes place entirely in the cytoplasm. Once the viral RNA enters the cytoplasm it is translated to produce the viral RNA-dependent RNA polymerase which then makes a full-length complementary (minus strand) copy

of the virion RNA. The minus strand serves a template for transcription of the seven capped and polyadenylated mRNAs. These are arranged as a nested set in which all have the same 3' end but each is smaller by one gene than the next larger one. All have the same 5' end, a 72 nucleotide leader sequence, encoded only at the 5' end of the genome RNA. This suggests each mRNA is  
5 transcribed by a mechanism in which transcription starts by synthesizing the leader sequence and then "skips" to the beginning of one of the genes with each mRNA ending at the same 3' end. Only the first gene (the one closest to the 5' end) is translated on each mRNA regardless of how many genes are present. Thus, there is no polyprotein processing in coronavirus replication.

Full-length genomic RNA molecules are synthesized from the minus strand, and these  
10 interact in the cytoplasm with N-protein forming the virus nucleocapsid. The nucleocapsid then buds into a cytoplasmic vesicle whose membrane contains the virus glycoproteins, forming mature virions. These vesicles can come to contain many virions. Virions exit the cell when the vesicle fuses with the cytoplasmic membrane. Coronaviruses do not mature by budding from the cytoplasmic membrane as many viruses do.

15 Coronaviruses exhibit a high rate of genetic recombination and mutation with deletion mutations being particularly common. Transcriptional skipping may be related to the high rate of recombination and mutation, but the exact mechanism is not yet known.

Viruses with positive-sense ssRNA genome (except the retroviruses) deliver their genomic RNA directly to cellular ribosomes and begin the infectious cycle with translation.  
20 These genomic RNAs are infectious even when completely deproteinized. Positive-sense RNA viruses fall into two groups: those that produce subgenomic mRNAs and those that do not produce subgenomic mRNAs.

Coronaviruses are a major cause of common colds in the winter months. The virus is found throughout the world, and is spread in respiratory secretions. Antibodies begin to appear in  
25 childhood, and are found in more than 90% of adults. The frequency of coronavirus respiratory infections is highly variable from year to year. The highest incidence occurs in years when rhinovirus colds are lowest. Coronavirus colds tend to occur in defined outbreaks. Laboratory diagnosis is by ELISA, complement fixation or hemagglutination tests. Human coronaviruses cannot be isolated by growth in culture.

30 Colds due to coronaviruses cannot be distinguished clinically from rhinovirus colds. The incubation period is 2-5 days and symptoms last 5-7 days. Immunity is directed to the major virus surface glycoprotein, E2. Re-infection may last for several years although reinfection is common probably because of the high level of virus genetic recombination.



In late 2002, several hundred cases of an atypical pneumonia were reported in Guangdong Province of the People's Republic of China. Months later, similar cases were identified in Canada, Vietnam and Hong Kong. The World Health Organization (WHO) identified the emergent disease as "severe acute respiratory syndrome" or SARS. By late April 5 2003, over 4300 SARS cases, resulting in about 250 deaths, were reported from 25 countries globally.

It is now possible to generally describe the course of the disease. The incubation period following initial infection is about 2 to 7 days. The infection is generally characterized by fever, which is followed in the next few days by dry, non-productive cough and shortness of breath. 10 The disease results in death in about 3 to 10% of cases.

The precise route of infection is not precisely known at present, although SARS CoV seems to populate the deep lung, at least in the more morbid forms of SARS. Elsewhere, SARS CoV has been denominated the "SARS associated coronavirus," or SCV). Whether the route of infection is via direct colonization of the deep lung, by initial nasal infection and subsequent 15 downward migration, via penetration to the blood stream and reemergence in the lung, or some other process is not currently known. However, the severe flu-like symptoms of the syndrome suggest high concentrations of virus in the deep lung, where it causes alveolar damage that is fatal in a high percentage of cases. What is known, however, is that the virus is transmitted almost entirely by large droplets reaching the mucous membranes of nose and lungs, and not by 20 other means.

Laboratories in the United States, Canada, Germany and Hong Kong have now isolated the causative viral agent, denominated "SARS-CoV" (for SARS coronavirus), and have obtained its genomic sequence. The evidence of SARS-CoV being the etiological agent in SARS is supported by multiple independent sources. Ksiazek et al., N. Eng. J. Med., 2003, 348, 1953-66; 25 Peiris et al., Lancet, 2003, 361, 1319-25; and Drosten et al., N. Eng. J. Med., 2003, 348, 1967-76.

In March 2003, a novel coronavirus (SARS-CoV) was discovered in association with cases of SARS. The complete genome of SARS CoV has been identified, as well as common variants thereof. The genome of SARS-CoV is 29,727 nucleotides in length and has 11 open 30 reading frames. Phylogenetic analyses and sequence comparisons showed that SARS-CoV is not closely related to any of the previously characterized coronaviruses. 41% of the residues of the genome are G or C. The genomic organization is typical of coronaviruses, with the characteristic gene order of 5'-replicase (rep), spike (S), envelope (E), membrane (M), nucleocapsid (N)-3' and

short untranslated regions at both termini. The SARS-CoV rep gene, which comprises about two-thirds of the genome, is predicted to encode two polyproteins that undergo co-translational proteolytic processing. There are four open reading frames (ORFs) downstream of rep that are predicted to encode the structural proteins, S, E, M, and N, which are common to all known  
5 coronaviruses. The hemagglutinin-esterase gene, which is present between ORF1b and S in group 2 and some group 3 coronaviruses was not found.

In the first stage of SARS infection, characterized by the patient running a high fever, ribavirin has been administered to reduce viral load, while in the second stage of infection, characterized by severe inflammation in the lung, steroids have been used to reduce  
10 inflammation. These therapies, however, are of limited utility, and do not address the underlying cause of SARS, which is unchecked viral replication. These therapies also do not offer any effective treatment for those who progress to late-stage SARS, characterized by extensive pulmonary destruction.

While it is currently believed that SARS CoV is the primary etiological agent for SARS,  
15 it is not known whether other infective agents, such as viruses, may be responsible for higher virulence, morbidity and/or mortality rates. There may also be a genetic component to morbidity and mortality, as it has been shown in some cases that related patients seem to have similar clinical outcomes. While these possibilities cannot be ruled out, it is accepted that reducing viral load in the lungs will correlate with improved prognosis.

20 Nucleosides and their derivatives have been used successfully in treatment of some viral infections, however to date no effective antiviral therapy has been identified for SARS, especially in the late stages.

Antisense agents, such as antisense oligonucleotides, PNAs, LNAs and morpholinos have been used to treat a variety of disease states. The first FDA-approved antisense drug is a  
25 phosphorothioate oligonucleotide (Vitravene®, fomivirsen) available through Isis Pharmaceuticals, Inc., Carlsbad, California. Fomivirsen is an antiviral antisense compound effective for treating CMV retinitis. Although it has been theorized to treat other viruses, e.g. hepatitis C, with antisense drugs, it has not been previously suggested to treat coronaviruses, and especially SARS CoV by inhalation of one or more antiviral compounds.

30 Methods of delivering drugs by pulmonary administration have been described. For example, each of U.S. Patent Nos. 6,550,472, 6,546,927, 6,543,443, 6,540,154, 6,540,153, 6,467,476, 6,427,682, 6,503,480, 6,447,753, 6,387,390, 5,985,320, 5,985,309, 5,855,913, 6,431,167, 6,408,854, 6,349,719, 6,167,880, 6,098,620, 5,971,951, 5,957,124, 5,906,202,

5,819,726, 5,755,218, 5,522,385, 6,546,929, 6,543,448, 6,509,006, 6,423,344, 6,303,582, and 6,138,668 teaches methods and devices useful in the pulmonary administration of drugs and/or nasal instillation. None of these U.S. patents, however, have demonstrated successful treatment of SARS by inhalation therapy.

5 Bioadhesives have been described for facilitating transport of medicaments across endothelial mucosa. For example, U.S. Patent No. 6,228,383 teaches use of bioadhesive fatty acid esters for facilitating transport of drug substances across mucosa in the lung, nose and other tissues.

Penetration enhancers have been described in, for example, U.S. Patent Application  
10 09/315,298, filed on May 20, 1999. Penetration enhancers facilitate the penetration of mucosa, including pulmonary and nasal mucosa.

The present invention provides, *inter alia*, compositions formulated for pulmonary or nasal administration of antiviral compounds, especially compounds capable of attenuating, mitigating or preventing coronavirus infections, and especially SARS CoA, the suspected  
15 etiological agent of SARS.

In some embodiments of the invention, SARS is treated by administering an antiviral antisense agent, such as an antiviral antisense oligonucleotide, to a patient in need thereof. In some embodiments of the invention, SARS is treated by administering an antiviral small molecule to a patient in need thereof. In some embodiments according to the present invention,  
20 an antiviral agent is administered by pulmonary or intranasal means to a patient in need thereof. In certain embodiments, the antiviral agent is an antisense agent. In other embodiments, the antiviral agent is a small molecule. In other embodiments of the invention, the antiviral agent is a mixture of antiviral antisense agent and an antiviral small molecule.

In some embodiments of the present invention, the inventive composition comprises, in  
25 addition to one or more antiviral agents, a therapeutically acceptable agent for intrapulmonary or intranasal administration.

## SUMMARY OF THE INVENTION

The present invention provides methods of treating a coronavirus infection comprising  
30 contacting a mammal having the coronavirus infection with an oligomeric compound, or composition comprising the same, wherein the oligomeric compound comprises at least one antiviral nucleoside and/or nucleoside mimetic. In some embodiments, the composition comprising the oligomeric compound is in a form suitable for administration via a pulmonary or

nasal route. In some embodiments, the oligomeric compound is an antisense compound or an siRNA. In some embodiments, the composition comprises: water, an isotonic saline solution, a buffered saline solution, one or more bioadhesives, one or more penetration enhancers, a fatty acid penetration enhancer (such as sodium laurate or sodium caprate), a liposome, a colloidal  
5 suspension, a second antiviral compound, an immune stimulatory agent, and/or an emulsifier, or any combination thereof. In some embodiments, the composition is a dry powder formulation, is present in a dropper suitable for instilling drops of composition to the nose, or is present in a device suitable for delivering single metered doses to the lung.

## 10 DESCRIPTION OF EMBODIMENTS

The present invention provides, *inter alia*, compounds, compositions and methods for treatment of viral infections and, in particular, coronavirus infections. In particular, the present invention relates to the treatment of coronavirus infections by administering to a patient in need thereof a therapeutically or prophylactically effective amount of an antiviral agent by inhalation  
15 or intranasal administration.

In terms of the present invention, inhalation includes all modes of instillation of drug, medicament or pharmaceutical composition to the lung(s). Included are nebulization, metered dosing, aerosolization, and the like.

In terms of the present invention, intranasal administration includes all modes of  
20 instilling a drug, medicament or pharmaceutical composition into the nose, including the nasal sinuses. Included are dropper, spraying, squirting, and the like.

The present invention provides compositions and methods of use for treating a coronavirus infection, and especially a SARS CoV infection. In the context of this invention, the terms "treatment", "treating", "treating SARS", "treating SARS CoV", "treating a coronavirus",  
25 "treating a coronavirus infection", "treating a SARS CoV infection", and "treating SCV," each indicates treatment of a mammal, in particular a human, patient with an amount of a composition sufficient to protect the patient from infection by SARS CoV (i.e. prophylaxis), to reduce the severity of SARS CoV infection (e.g. by directly or indirectly interfering with the ability of the virus to replicate, assemble, infect host tissue, or cause damage to the host tissue), including to  
30 cure the patient of the viral infection, and also including treating and/or ameliorating one or more symptoms of the infection. In this context, treatment does not necessarily include curing the patient, but may include merely reducing the severity of symptoms attendant to the disease,

reducing viral load until other, more effective antiviral therapies are employed, or the patient's immune system is able to clear the virus, or until the patient eventually succumbs.

Compositions according to the present invention include one or more antiviral nucleosides as and/or nucleoside mimetics as set forth herein, one or more antiviral dimers as set  
5 forth herein, or antiviral antisense agents as described herein and elsewhere. More specifically, antiviral antisense compounds according to the present invention are compounds able to specifically bind to and interfere with the expression of one or more viral nucleic acids of SARS CoV, or one or more nucleic acids intracellularly derived from SARS CoV. Antiviral compounds according to the present invention may be prepared in a variety of formulations suitable for nasal  
10 or pulmonary delivery, as described in more detail herein.

As used herein, the term "antisense compound," as well as its plural form, is intended to embrace the full scope of compounds capable of specifically binding via, *inter alia*, Watson and Crick base pairing, to a stretch of nucleic acid, and to modulate expression of the nucleic acid to which it is bound. Such modulation may include up- or down-regulation of expression of the  
15 nucleic acid. Antisense compounds include, but are not limited to, nucleic acids, e.g. all-phosphodiester, all-phosphorothioate, or mixed phosphodiester and phosphorothioate oligonucleotides. Antisense compounds also include, but are not limited to, oligonucleotides containing one or more non-phosphate linkages between adjacent sugars. Also, antisense compounds include, but are not limited to, oligonucleotides in which one or more of the sugars  
20 are modified. Exemplary modifications include, but are not limited to, 2'-(optionally substituted)-O-alkyl substituents, locked nucleic acids (in which the 2'- and 4'-positions of a nucleoside are bridged), 2'-substituted deoxy nucleosides, and the like. In addition, antisense compounds include oligonucleotides containing one or more modified bases, such as 5-methylcytosine (5-MeC), as well as 5-propynyl pyrimidines, and the like. Also, antisense compounds include, but  
25 are not limited to, oligomers containing one or more non-nucleoside backbones, such as morpholinos or peptide nucleic acids.

Antisense compounds according to the present invention are capable to specifically binding one or more nucleic acids that either originate in the SARS CoV virion or are produced by natural biological processes within cells of the host. Thus, antisense compounds may bind  
30 directly to the plus strand, the minus strand, or one of the mRNAs synthesized in the cell cytoplasm. While not wishing to be bound by theory, it is anticipated that an antisense compound directed to a plus-strand will effectively interfere with both minus strand biosynthesis and translation of mRNA, as such an antisense compound will, it is believed, bind to both genomic

plus strand RNA and mRNA. In some embodiments, where an RNase H mechanism is employed (e.g., where part or all of the antisense agent is DNA-like), it is theorized that gene silencing may arise out of specific cleavage of the genomic RNA, as well as cleavage of targeted mRNA. In other embodiments, e.g. where an siRNA mode of action is employed, cleavage may  
5 be at either the plus strand RNA, the minus strand genomic RNA or one or more mRNAs. In other embodiments, such as translational arrest modes of action, one or more splice sites may be targeted, on either the plus or minus strands, or advantageously at or within about 20 bases of the transcriptional or translational start codon. Other modes of action are possible and are considered within the scope of the present disclosure.

10 Compositions according to the present invention may be applied to either the lung or nasal passages by a variety of methods. The compositions may be applied to the lung, for instance, by use of dry powder inhalers or metered dose inhalers. The compositions may be applied to the paranasal sinuses by spraying or instilling the composition into one or both nostrils.

15 Compositions according to the present invention comprise an antisense compound or a nucleoside or nucleoside mimetic, optionally in combination with one or more additional ingredients, which may or may not have antiviral activity. Compositions according to the invention may be solids, liquids, multiphasic mixtures (e.g. emulsions), including microemulsions and creams, and related formulations, such as liposomes. Exemplary  
20 embodiments of the present invention include, but are not limited to, saline solution, buffered or unbuffered, oil-in-water emulsions, water-in-oil emulsions, oil-in-water-in-oil emulsions, and water-in-oil-in-water emulsions.

The methods according to the present invention may operate via either systemic or local modes. While not wishing to be bound by theory, the compositions according to the present  
25 invention are capable penetrating cells that are the targets of viral infection, wherein the antiviral compounds according to the present invention interfere with one or more functions of cell replication, assembly or cell exit. It is believed that the locus of activity is in the lung or nasal passages, however it is also possible that some quantity of the compound according to the present invention is able to cross the nasal or pulmonary epithelium into the blood stream, where  
30 it becomes available to bring about systemic antiviral activity. It may be that a combination of local and systemic antiviral activity are at work in the present methods, and the present inventors do not wish to be bound by any particular mode of action, provided that a pulmonary or nasal route of administration is required.

In some embodiments according to the present invention, the antiviral compound according to the invention may operate by more than one biological pathway. For example, while it is desirable to design antisense compounds that interfere with expression of a viral nucleic acid, it is also possible to design an antisense compound that is capable of stimulating the patient's immune system, whereby it may be possible to enhance the patient's ability to raise an immune response to the viral infection. It has been previously found that the CpG motif (CG) is especially useful for stimulating the immune complement system. While not wishing to be bound by theory, it is believed that the CpG motif works by stimulating Tol-like-receptor 9 (TLR-9), giving rise to non-specific immune stimulation. In some embodiments of the invention, especially where prophylaxis is desired (e.g., the viral titer is low or the patient is suspected of having been infected with the virus but has yet to seroconvert and it is desired to prevent or preemptively reduce the severity of viral infection) it may be beneficial to design an antisense compound (e.g., a phosphorothioate oligonucleotide) having one or more CG subsequences. In other embodiments, e.g. where the patient's lungs are fully involved, it may be desirable to avoid the CG motif so as to avoid exacerbation of already present pulmonary edema.

Compositions according to the present invention may incorporate one or more bioadhesives, which increase bioavailability across mucosa, including nasal, oropharynx, nasopharynx, and hypopharynx, and pulmonary mucosa.

Compositions according to the present invention may also comprise one or more penetration enhancers. Exemplary penetration enhancers for use in the present invention include, but are not limited to fatty acids, bile salts, chelating agents, surfactants, and non-surfactants.

Compositions according to the present invention may comprise one or more mucolytic agents as well.

Compositions according to the present invention may be formulated in the form of a liposome. Suitable liposomes are known in the art. In some embodiments of the invention, the liposomal formulation is in a form suitable for pulmonary instillation, e.g. for inhalation. In other embodiments, the liposomal formulation is in a form suitable for intranasal instillation, e.g. for spraying or administering via dropper to the nose. In particular embodiments according to the present invention, the liposome further comprises Tat peptide. Liposomes may be instilled into the lung as taught in U.S. Patent No. 5,049,388.

Compositions according to the present invention may be delivered in the form of liquids or solids. While intratracheal or endoscopic instillation of a liquid composition of the present invention is possible, and is within the scope of the present invention, patient compliance is

predicted to be low for such methods. Nebulization of a liquid composition is also a possibility, and is also within the scope of the present invention. In some embodiments of the invention, the compositions according to the present invention are prepared as liquid solutions or mixtures and are instilled via intratracheal or endoscopic instillation, or are nebulized using a standard  
5 nebulizer known in the art. Typical volumes of compositions administered via these routes average between about 0.1 to about 10 ml. In general, smaller volumes are suitable.

Compositions according to the present invention, whether solid or liquid, may also be aerosolized via an art recognized method and delivered to the lung in a single breath or a series of breaths.

10 Delivery of compounds and compositions described herein are described in, for example, International Publications WO 99/60167, WO 99/60166, and WO 99/01579.

Delivery of dry powder nucleic acid compositions to the lung have been described in U.S. Patent No. 6,303,582. The compositions according to the present invention are formulated in a similar manner, except that in most cases the nucleic acid is replaced with an antisense  
15 compound. As discussed herein, some nucleic acids may act as antisense compounds under certain circumstances. In general, however, the term "nucleic acid" as used in U.S. Patent No. 6,303,582 does not apply to antisense compounds, as antisense compounds as disclosed herein generally contain one or more chemical modifications that stabilize the antisense compound against attack by nucleases, and are generally unsuitable for use in transfecting cells with  
20 expressible genetic material. In particular, antisense compounds generally contain one or more non-phosphodiester backbone modification (e.g. phosphorothioate, phosphoramidate, MMI, etc.). In addition, antisense compounds may also contain one or more base or sugar modifications. Such modifications are discussed in more detail hereinafter.

In some embodiments, the compositions according to the present invention may be  
25 compounded as dry particles having mean diameters in the range of about 0.5 to about 200  $\mu\text{m}$ , e.g. in the range of about 0.5 to about 5  $\mu\text{m}$ , and advantageously in the range of about 1 to about 5  $\mu\text{m}$ . In other embodiments, the dry particles may be compounded as aerodynamically light particles in the range of about 5  $\mu\text{m}$  to about 50  $\mu\text{m}$ , especially in the range of about 5 to about 30  $\mu\text{m}$ , e.g. about 5 to about 15  $\mu\text{m}$ . See U.S. Patent No. 6,503,480. In general, it is desirable for  
30 particles to have an aerodynamic mass median diameter in the range of from about 1 to about 3  $\mu\text{m}$ . See U.S. Patent No. 5,049,388. Such criteria can be met by aerodynamically light solid particles as described above, by liposomes, by blends of particles containing compositions



according to the present invention with excipients, including suspending agents, etc. See U.S. Patent No. 6,433,040.

In some embodiments, the compositions according to the present invention may be compounded as a liquid formulation that is delivered by nebulization, single metered dosing, etc.

5 In general, it is desirable for particles to have an aerodynamic mass median diameter in the range of from about 1 to about 3  $\mu\text{m}$ . See, U.S. Patent Nos. 5,049,388 and 6,309,623.

Compositions according to the present invention, whether solid or liquid, may contain one or more excipients, adjuvants or other pharmaceutically acceptable compounds. In the context of this invention, the term "admixant" will be used to refer to all pharmaceutically  
10 acceptable compounds that may be combined with anti-SARS antisense compounds, anti-SARS nucleosides, anti-SARS nucleoside mimetics, or anti-SARS dimers as described herein. In some embodiments, the compositions of the present invention comprise one or more surfactants. Additional admixants include, but are not limited to, penetration enhancers, cell-permeation enhancers, salts, compounds capable of recognizing and directing the particles to a particular  
15 cell-surface receptor, and the like.

The dry particles of compositions according to the present invention may be prepared according to art-accepted methods. In some embodiments according to the present invention, the antisense compounds may be pre-treated with supercritical carbon dioxide to remove residual organic solvents, etc., as well as to prepare the antisense compound in a form suitable for  
20 preparing small particles. For example, phosphorothioate backbone oligonucleotides may be purified by supercritical carbon dioxide extraction. The thus-purified phosphorothioate oligonucleotides may then be dissolved in a suitable solvent, such as water, and combined with a suitable hydrophilic excipient. This solution may then be dried by spray drying, vacuum drying, or some other suitable method to produce a crude powder, which may then be ground to form a  
25 particle mixture of the desired size and density.

In some embodiments according to the present invention, the nucleic acid may be prepared in the form of liposomes. In some embodiments, the liposome solutions may then be dried as described herein. In some embodiments according to the present invention, the liposome solutions may be prepared substantially free of salts and/or buffers, thereby enhancing the ability  
30 of antisense agents to cross cell membranes.

In some embodiments of the invention, the compositions are formulated as low density particles. Suitable low density particles are described in U.S. Patent No. 6,503,480. In some embodiments, compositions according to the present invention are formulated as

aerodynamically light particles comprising a biodegradable material having a tap density of between 0.1 and 1.4 g/cm<sup>3</sup>, especially about 0.4 g/cm<sup>3</sup>, and a mean diameter of about 5 µm to about 30 µm. Such compositions may be aerosolized for delivery to the deep lung.

In some embodiments, the compositions according to the present invention comprise one or more permeating enhancers. The permeation enhancers may be chosen to enhance permeation of the pulmonary or nasal mucosa, to enhance penetration of endothelial, alveolar or basal cell membranes, to enhance endocytosis by target cells, and the like.

Phagocytosis of particles delivered to the lung has been found to greatly diminish the efficacy of inhaled drugs. It has been found that liquid particles greater than about 3 µm in diameter, or less than about 1 mm in diameter, are not recognized by phagocytes in the lung, and are, thus, not subject to phagocytosis. Accordingly, in some embodiments of the present invention, the compositions will be aerosolized to achieve a mean particle size of greater than about 3 µm in diameter. In other embodiments, it is desirable that the mean particle size be less than about 1 µm in diameter. In some embodiments of the invention, the particle size is greater than about 3 µm in diameter, e.g. in the range of about 3 to about 10 µm in diameter. The mean particle density is advantageously in the range of about 1 g/cm<sup>3</sup>.

Antisense compounds include oligomers as described herein below that elicit an antisense effect, whether by RNase H activation, siRNA, splice site junction interference, translational arrest, and the like.

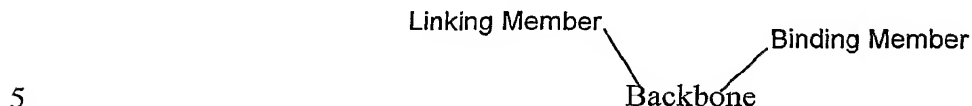
## *Oligomers*

In the context of the invention, the terms "oligomeric compound" and "oligomer" refer to a polymeric structure capable of selectively binding to (hybridizing to) a sequence of nucleic acid molecule (e.g. DNA, RNA or derivative). Each oligomer comprises a plurality of monomer subunits joined together by linking members. Each monomer subunit comprises at least a binding member, a linking member and optionally a backbone member. The function of the binding member is to provide sequence-specific binding to a target oligo- or polynucleotide. These interactions may be Watson-Crick hybridization, Hoogsteen base pairing, a combination of these mechanisms, or some other sequence-specific interaction.

The function of the backbone member is to hold the binding members in a spatial configuration amenable to sequence-specific binding, while also providing structural support for the linking member. The backbone member reduces unfavorable entropic effects on binding by holding the binding members of an oligomer in a configuration that is amenable to sequence-

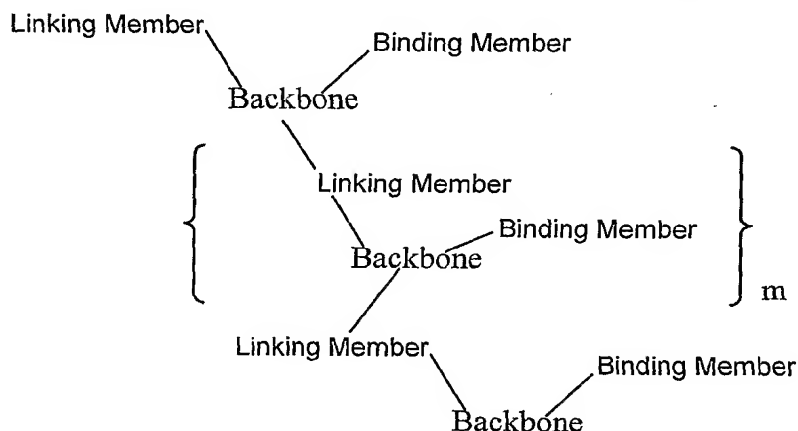
specific binding. In general, the backbone member is covalently attached to the linking member or members and the binding member.

A subunit of an oligomer is schematically depicted hereinafter, wherein Binding Member, Linker and Backbone are as described herein.



A linking member of one subunit is covalently linked to an adjacent subunit. Typical linking members are described in more detail below, as are binding members and backbone members. An oligomer is formed when multiple subunits are concatenated to form a chain or sequence. A typical n-mer oligomer is depicted below, wherein Linking Member, Backbone and Binding Member are described herein, n is m+2, and m is 0 or an integer.

10



In typical oligomers according to the present invention, n is from about 8 to about 80, more specifically from about 8 to about 40, and even more specifically from about 8 to about 25. In particular embodiments of the invention, n has one of the values 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25.

15

### *Oligonucleotides*

The basic subunit of an oligonucleotide, such as RNA or DNA is depicted below.

5 by condensation with the 5'-OH of an adjacent subunit, thereby forming a phosphate diester bond. Where each of G' and G'' is O, this is called a phosphodiester bond; where one of G' or G'' is S and the other is O, this is called a phosphorothioate bond, and where both G' and G'' are S, this is called a phosphorodithioate bond.

10 DNA (deoxyribonucleic acid) and OH for RNA (ribonucleic acid), each of G' and G'' is O and Bx is one of the following structures:



wherein G, C, A, U and T are guanine, cytosine, adenine, thymine and uracil, respectively.

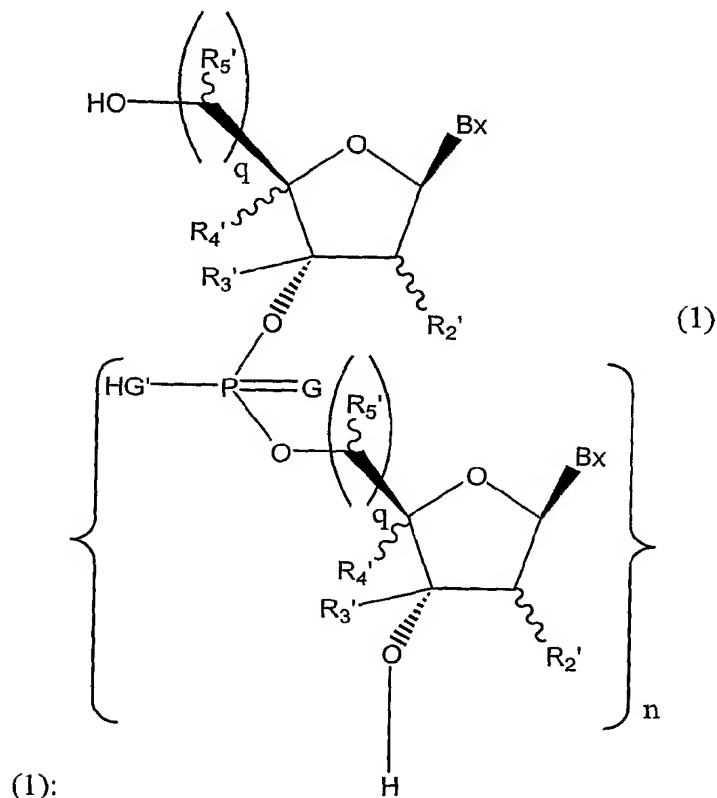
In the above formula, G' and G'' may be O or S, and R<sub>2</sub>' may be H, OH or some other value.

25 C, A and U, and the backbone comprises a sugar residue (ribosyl, i.e.  $R_2'$  is OH) and a phosphate ( $G' = G'' = O$ ). The ribosyl sugar residue is the backbone member, while the phosphate joins adjacent monomers through the 5'- and 3'- oxygen atoms on the ribosyl ring. The sugar is

covalently bound to the nucleosidic base (base) at the 1'-position, the  $-\beta$ -D configuration predominating.

Naturally occurring DNA is analogous to RNA, except that the sugar is a 2'-deoxyribosyl ( $R_2'$  is H).

5 Generally oligonucleotides according to the present invention include naturally occurring and non-naturally occurring oligonucleotides. In general, oligonucleotides according to the present invention include compounds of the formula



wherein:

- 10 each Bx is a nucleobase as defined herein;  
 each q is 0 or 1;  
 each of  $R_2'$  is H, OH, reversibly-protected OH or a substituent or together with  $R_4'$  forms a bridge;  
 $R_3'$  is H or a substituent;  
 15  $R_4'$  is H, a substituent or together with  $R_2'$  or  $R_5'$  forms a bridge;  
 $R_5'$  is H, a substituent or together with  $R_4'$  forms a bridge, and each squiggly bond (~~~~) indicates that the bond may be in the up or down configuration.

The naturally occurring oligonucleotides are those in which each of Bx is selected from the group consisting of G, C, A, U (for RNA) and T (DNA), each of G' and G'' is O, each R<sub>3</sub>', each R<sub>4</sub>', each R<sub>5</sub>' is H, each q is 1 and n is an integer, and the sugar oxygens are in the ribosyl configuration. Conversely, non-naturally occurring oligonucleotides include those in which at  
5 least one of the following conditions applies: at least one Bx is a nucleobase other than G, C, A, U (for RNA) and T (DNA), at least one of G' and G'' is other than O, at least one R<sub>3</sub>', R<sub>4</sub>', or R<sub>5</sub>' is other than H, at least at least one q is 0, or at least one of the sugar oxygens is in other than the ribosyl configuration.

As used herein, the term "oligonucleotide" encompasses both naturally occurring  
10 oligonucleotides and non-naturally occurring oligonucleotides, or mixtures thereof. In specific embodiments of the present invention, the term oligonucleotide refers to a non-naturally occurring oligonucleotide having both naturally-occurring and non-naturally-occurring nucleotide subunits. In specific embodiments of the invention, one or more nucleobases, sugar backbones and/or phosphate linking members are non-naturally-occurring. These features will be  
15 described in greater detail below.

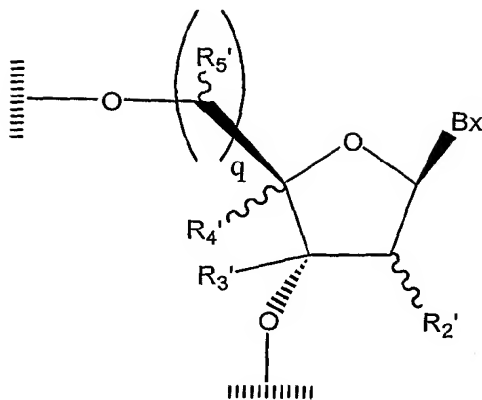
In general, hybridization may occur between a single-stranded oligonucleotide and a single- or double-stranded intracellular nucleic acid, or between a double-stranded oligonucleotide and a single- or double-stranded intracellular nucleic acid. Additionally, oligonucleotides may be prepared as double-stranded oligonucleotides, which then may be  
20 unwound by intracellular agents (e.g. helicase), after which the antisense strand may hybridize a target nucleic acid (e.g. mRNA), thereby giving rise to an antisense effect (e.g. cleavage of the mRNA strand by the RISC complex). In this context, the term oligonucleotide includes both single- and double-stranded oligonucleotides, and it is understood that where a single strand is disclosed herein, the complementary (sense) strand is also disclosed by inference. In some cases,  
25 the double stranded oligonucleotide comprises at least one strand in which all the sugars are ribosyl, 2'-deoxy-2'-fluoroarabinosyl, or 2'-deoxy-2'-fluororibosyl, or mixtures thereof. In some cases, the double stranded oligonucleotide comprises at least one strand in which one or more of the phosphodiester linkages are replaced with phosphorothioate linkages. In some cases, no more than half of the internucleoside linkages are phosphorothioate linkages.

30

### *Sugar Backbone*

In general, the sugar backbone has the structure:

- 18 -



wherein:

each Bx is a nucleobase as defined herein;

q is 0 or 1;

5 each of R<sub>2</sub>' is H, OH, reversibly-protected OH or a substituent or together with R<sub>4</sub>' forms a bridge;

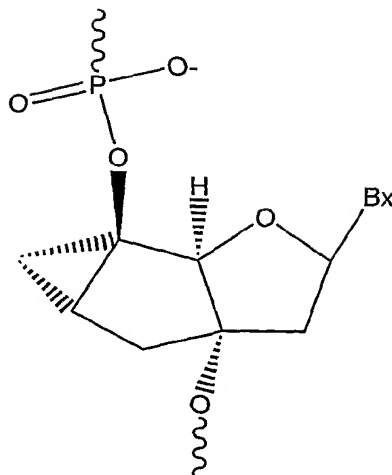
R<sub>3</sub>' is H or a substituent;

R<sub>4</sub>' is H, a substituent or together with R<sub>2</sub>' or R<sub>5</sub>' forms a bridge;

10 R<sub>5</sub>' is H, a substituent or together with R<sub>4</sub>' forms a bridge. The dashes (————) indicate the positions at which the sugar moiety is bound to a phosphate linker to form a nucleotide bond.

The person skilled in the art will recognize that when R<sub>2</sub>' is in the down configuration and q' is 1, the ring is a ribosyl ring, whereas when R<sub>2</sub>' is in the up configuration and q' is 1, the ring is an arabinosyl ring. Likewise, when q' is 0 and R<sub>2</sub>' is in the down configuration, the ring is an erythrosyl ring. When R<sub>2</sub>' and R<sub>4</sub>' are joined to form a bridge, the ring is called a locked  
 15 nucleic acid (LNA), as described in greater detail herein. In some embodiments, the bridge formed by R<sub>2</sub>' and R<sub>4</sub>' is R<sub>2</sub>'-O-(CH<sub>2</sub>)<sub>r</sub>-R<sub>4</sub>' (wherein r is 1 or 2) or R<sub>2</sub>'-CH<sub>2</sub>-O-CH<sub>2</sub>-R<sub>4</sub>' (the use of R<sub>2</sub>' and R<sub>4</sub>' in the sub-formulae indicating the points of attachment). LNA may be present in either α-L- or β-D- conformation. See Vester et al., "LNAszymes: Incorporation of LNA-Type Monomers into DNAszymes Markedly Increases RNA Cleavage," J. Amer. Chem. Soc., 2002,  
 20 124, 13682-3. Each of these analogs possesses a number of useful characteristics, including resistance to exonuclease activity, induction of endonuclease activity (e.g. by RNase H, the RISC complex, etc.) and modulation of hybridization.

When R<sub>4</sub>' and R<sub>5</sub>' form a bridge, they may form, along with the sugar ring to which they are attached, a tricyclic ring. Tricyclic nucleosides of the structure:



are described by Rennenberg et al. in *Nucleic Acids Research*, 2002, 30(13), 2751-7. One skilled in the art will recognize that the analogous phosphorothioates, and 2'-substituted tricyclic deoxynucleosides may be prepared by methods analogous to those taught by Rennenberg et al., as modified by the teaching herein. In particular, the phosphorothioates may be prepared by substituting a sulfurizing oxidant (a.k.a. a sulfur transfer reagent, such a phenyl acetyl disulfide) for the oxidizing agent taught by Rennenberg et al. The 2'-substituted tricyclic deoxynucleosides may be prepared from the analogous 2'-substituted deoxynucleosides, using a 2'-OH protecting group in the case of ribonucleic acid.

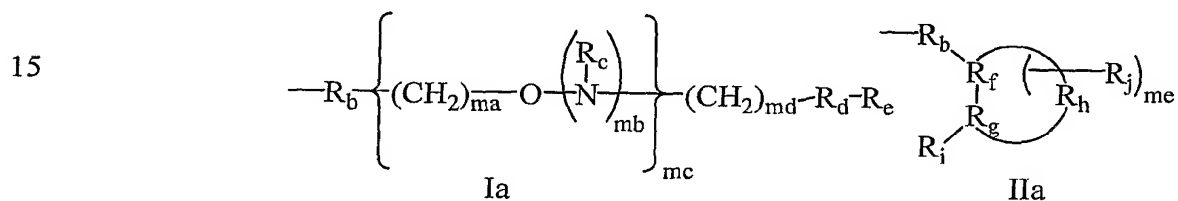
10 Certain oligonucleotides that utilized arabino-pentofuranosyl nucleotides as building blocks have been described. Damha et. al., *J.A.C.S.*, 1998, 120, 12976-12977; and Damha et. al., *Bioconjugate Chem.*, 1999, 10, 299-305.

Suitable 2'-substituents corresponding to  $R_2'$  include, but are not limited to, F, O-alkyl (e.g. O-methyl), S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl; O-alkynyl, S-alkynyl, N-alkynyl; O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted  $C_1$  to  $C_{10}$  alkyl or  $C_2$  to  $C_{10}$  alkenyl or alkynyl, respectively. Particularly suitable are  $O((CH_2)_gO)_hCH_3$ ,  $O(CH_2)_gOCH_3$ ,  $O(CH_2)_gNH_2$ ,  $O(CH_2)_gCH_3$ ,  $O(CH_2)_gONH_2$ , and  $O(CH_2)_gON((CH_2)_gCH_3)_2$ , where g and h are from 1 to about 10. Other suitable oligonucleotides comprise one of the following at the 2' position:  $C_1$  to  $C_{10}$  lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH<sub>3</sub>, OCN, Cl, Br, CN, CF<sub>3</sub>, OCF<sub>3</sub>, SOCH<sub>3</sub>, SO<sub>2</sub>CH<sub>3</sub>, ONO<sub>2</sub>, NO<sub>2</sub>, N<sub>3</sub>, NH<sub>2</sub>, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other



substituents having similar properties. One 2'-modification is 2'-deoxy-2'-methoxyethoxy (2'-O-CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>, also known as 2'-O-(2-methoxyethyl) or 2'-MOE ribosyl) (Martin et al., Helv. Chim. Acta, 1995, 78, 486-504). Other suitable modifications include 2'-dimethylaminoethoxyethoxy, i.e., a O(CH<sub>2</sub>)<sub>2</sub>ON(CH<sub>3</sub>)<sub>2</sub> group, also known as 2'-DMAOE, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'-O-CH<sub>2</sub>-O-CH<sub>2</sub>-N(CH<sub>3</sub>)<sub>2</sub>.

Other suitable modifications include, but are not limited to, 2'-methoxy (2'-O-CH<sub>3</sub>), 2'-aminopropoxy (2'-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>), 2'-allyl (2'-CH<sub>2</sub>-CH=CH<sub>2</sub>), 2'-O-allyl (2'-O-CH<sub>2</sub>-CH=CH<sub>2</sub>) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. A suitable 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Further representative substituent groups include groups of formula I<sub>a</sub> or II<sub>a</sub>:

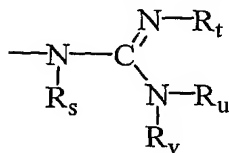


wherein:

R<sub>b</sub> is O, S or NH;

20 R<sub>d</sub> is a single bond, O or C(=O);

R<sub>e</sub> is C<sub>1</sub>-C<sub>10</sub> alkyl, N(R<sub>k</sub>)(R<sub>m</sub>), N(R<sub>k</sub>)(R<sub>n</sub>), N=C(R<sub>p</sub>)(R<sub>q</sub>), N=C(R<sub>p</sub>)(R<sub>r</sub>) or has formula III<sub>a</sub>;



25 III<sub>a</sub>

each R<sub>c</sub>, R<sub>q</sub>, R<sub>r</sub>, R<sub>s</sub>, R<sub>t</sub>, R<sub>u</sub> and R<sub>v</sub> is, independently, hydrogen, C(O)R<sub>w</sub>, substituted or unsubstituted C<sub>1</sub>-C<sub>10</sub> alkyl, substituted or unsubstituted C<sub>2</sub>-C<sub>10</sub> alkenyl, substituted or unsubstituted C<sub>2</sub>-C<sub>10</sub> alkynyl, alkylsulfonyl, arylsulfonyl, a chemical functional group or a conjugate group, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, 30 carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl;

or optionally, R<sub>u</sub> and R<sub>v</sub>, together form a phthalimido moiety with the nitrogen atom to which they are attached;

each  $R_w$  is, independently, substituted or unsubstituted  $C_1$ - $C_{10}$  alkyl, trifluoromethyl, cyanoethoxy, methoxy, ethoxy, t-butoxy, allyloxy, 9-fluorenylmethoxy, 2-(trimethylsilyl)-ethoxy, 2,2,2-trichloroethoxy, benzyloxy, butyryl, iso-butyryl, phenyl or aryl;

$R_k$  is hydrogen, a nitrogen protecting group or  $-R_x-R_y$ ;

5  $R_p$  is hydrogen, a nitrogen protecting group or  $-R_x-R_y$ ;

$R_x$  is a bond or a linking moiety;

$R_y$  is a chemical functional group, a conjugate group or a solid support medium; each  $R_m$  and  $R_n$  is, independently, H, a nitrogen protecting group, substituted or unsubstituted  $C_1$ - $C_{10}$  alkyl, substituted or unsubstituted  $C_2$ - $C_{10}$  alkenyl, substituted or unsubstituted  $C_2$ - $C_{10}$  alkynyl, 10 wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl, alkynyl;  $NH_3^+$ ,  $N(R_u)(R_v)$ , guanidino and acyl where said acyl is an acid amide or an ester;

or  $R_m$  and  $R_n$ , together, are a nitrogen protecting group, are joined in a ring structure that optionally includes an additional heteroatom selected from N and O or are a chemical functional 15 group;

$R_i$  is  $OR_z$ ,  $SR_z$ , or  $N(R_z)_2$ ;

each  $R_z$  is, independently, H,  $C_1$ - $C_8$  alkyl,  $C_1$ - $C_8$  haloalkyl,  $C(=NH)N(H)R_u$ ,  $C(=O)N(H)R_u$  or  $OC(=O)N(H)R_u$ ;

$R_f$ ,  $R_g$  and  $R_h$  comprise a ring system having from about 4 to about 7 carbon atoms or 20 having from about 3 to about 6 carbon atoms and 1 or 2 heteroatoms wherein said heteroatoms are selected from oxygen, nitrogen and sulfur and wherein said ring system is aliphatic, unsaturated aliphatic, aromatic, or saturated or unsaturated heterocyclic;

$R_j$  is alkyl or haloalkyl having 1 to about 10 carbon atoms, alkenyl having 2 to about 10 carbon atoms, alkynyl having 2 to about 10 carbon atoms, aryl having 6 to about 14 carbon 25 atoms,  $N(R_k)(R_m)$   $OR_k$ , halo,  $SR_k$  or CN;

$m_a$  is 1 to about 10;

each  $m_b$  is, independently, 0 or 1;

$m_c$  is 0 or an integer from 1 to 10;

$m_d$  is an integer from 1 to 10;

30  $m_e$  is from 0, 1 or 2; and

provided that when  $m_c$  is 0,  $m_d$  is greater than 1.

Representative substituent groups of Formula I are disclosed in, for example, International Publication WO 00/08042. Representative cyclic substituent groups of Formula II are disclosed in, for example, International Publication WO 00/06590.

Particularly suitable sugar substituent groups include, but are not limited to, 5  $O((CH_2)_gO)_hCH_3$ ,  $O(CH_2)_gOCH_3$ ,  $O(CH_2)_gNH_2$ ,  $O(CH_2)_gCH_3$ ,  $O(CH_2)_gONH_2$ , and  $O(CH_2)_gON((CH_2)_gCH_3)_2$ , where g and h are from 1 to about 10.

Some suitable oligomeric compounds of the invention contain at least one nucleoside having one of the following substituent groups:  $C_1$  to  $C_{10}$  lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH<sub>3</sub>, OCN, Cl, Br, CN, CF<sub>3</sub>, OCF<sub>3</sub>, SOCH<sub>3</sub>, 10 SO<sub>2</sub>CH<sub>3</sub>, ONO<sub>2</sub>, NO<sub>2</sub>, N<sub>3</sub>, NH<sub>2</sub>, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligomeric compound, or a group for improving the pharmacodynamic properties of an oligomeric compound, and other substituents having similar properties. A suitable modification includes 2'-methoxyethoxy (2'-O- 15 CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, 1995, 78, 486), i.e., an alkoxyalkoxy group. Another modification is 2'-dimethylaminoethoxy, i.e., a  $O(CH_2)_2ON(CH_3)_2$  group, also known as 2'-DMAOE. Representative aminooxy substituent groups are described in co-owned U.S. Patent Application Serial Number 09/344,260, filed June 25, 1999, entitled "Aminooxy-Functionalized Oligomers"; 20 and U.S. Patent Application Serial Number 09/370,541, filed August 9, 1999, entitled "Aminooxy-Functionalized Oligomers and Methods for Making Same."

Other suitable modifications include, but are not limited to, 2'-methoxy (2'-O-CH<sub>3</sub>), 2'-aminopropoxy (2'-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>), and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on nucleosides and oligomers, particularly the 3' position of the sugar on 25 the 3' terminal nucleoside or at a 3'-position of a nucleoside that has a linkage from the 2'-position such as a 2'-5' linked oligomer and at the 5' position of a 5' terminal nucleoside. Oligomers may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative U.S. patents that teach the preparation of such modified sugars structures include, but are not limited to, 4,981,957; 5,118,800; 5,319,080; 5,359,044; 30 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, and commonly owned U.S. Patent Application 08/468,037 filed on June 5, 1995.

Representative guanidino substituent groups that are shown in formula III and IV are disclosed in co-owned International Publication WO 01/02423.

Representative acetamido substituent groups are disclosed in U.S. Patent No. 6,147,200. Representative dimethylaminoethoxyethyl substituent groups are disclosed in International Patent Application PCT/US99/17895, entitled "2'-O-Dimethylaminoethoxyethyl-Modified Oligonucleotides", filed August 6, 1999. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. The respective ends of this linear polymeric structure can be joined to form a circular structure by hybridization or by formation of a covalent bond, however, open linear structures are generally suitable. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside linkages of the oligonucleotide. The normal internucleoside linkage of RNA and DNA is a 3' to 5' phosphodiester linkage.

While the present invention may be adapted to produce oligonucleotides for any desired end use (e.g. as probes for use in the polymerase chain reaction), one suitable use of the oligonucleotides is in antisense therapeutics. One mode of action that is often employed in antisense therapeutics is the so-called RNase H mechanism, whereby a strand of DNA is introduced into a cell, where the DNA hybridizes to a strand of RNA. The DNA-RNA hybrid is recognized by an endonuclease, RNase H, which cleaves the RNA strand. In normal cases, the RNA strand is messenger RNA (mRNA), which, after it has been cleaved, cannot be translated into the corresponding peptide or protein sequence in the ribosomes. In this way, DNA may be employed as an agent for modulating the expression of certain genes.

It has been found that by incorporating short stretches of DNA into an oligonucleotide, the RNase H mechanism can be effectively used to modulate expression of target peptides or proteins. In some embodiments of the invention, an oligonucleotide incorporating a stretch of DNA and a stretch of RNA or 2'-modified RNA can be used to effectively modulate gene expression. In some embodiments, the oligonucleotide comprises a stretch of DNA flanked by two stretches of 2'-modified RNA. Suitable 2'-modifications include 2'-O-methyl and 2'-O-methoxyethyl as described herein.

The ribosyl sugar moiety has also been extensively studied to evaluate the effect its modification has on the properties of oligonucleotides relative to unmodified oligonucleotides. The 2'-position of the sugar moiety is one of the most studied sites for modification. Certain 2'-

substituent groups have been shown to increase the lipophilicity and enhance properties such as binding affinity to target RNA, chemical stability and nuclease resistance of oligonucleotides. Many of the modifications at the 2'-position that show enhanced binding affinity also force the sugar ring into the C<sub>3</sub>-endo conformation.

5 RNA exists in what has been termed "A Form" geometry while DNA exists in "B Form" geometry. In general, RNA:RNA duplexes are more stable, or have higher melting temperatures (T<sub>m</sub>) than DNA:DNA duplexes (Sanger et al., *Principles of Nucleic Acid Structure*, 1984, Springer-Verlag; New York, NY.; Lesnik et al., *Biochemistry*, 1995, 34, 10807-10815; Conte et al., *Nucleic Acids Res.*, 1997, 25, 2627-2634). The increased stability of RNA has been  
10 attributed to several structural features, most notably the improved base stacking interactions that result from an A-form geometry (Searle et al., *Nucleic Acids Res.*, 1993, 21, 2051-2056). The presence of the 2' hydroxyl in RNA biases the sugar toward a C3' endo pucker, i.e., also designated as Northern pucker, which causes the duplex to favor the A-form geometry. On the other hand, deoxy nucleic acids prefer a C2' endo sugar pucker, i.e., also known as Southern  
15 pucker, which is thought to impart a less stable B-form geometry (Sanger, W. (1984) *Principles of Nucleic Acid Structure*, Springer-Verlag, New York, NY). In addition, the 2' hydroxyl groups of RNA can form a network of water mediated hydrogen bonds that help stabilize the RNA duplex (Egli et al., *Biochemistry*, 1996, 35, 8489-8494).

DNA:RNA hybrid duplexes, however, are usually less stable than pure RNA:RNA  
20 duplexes, and depending on their sequence may be either more or less stable than DNA:DNA duplexes (Searle et al., *Nucleic Acids Res.*, 1993, 21, 2051-2056). The structure of a hybrid duplex is intermediate between A- and B-form geometries, which may result in poor stacking interactions (Lane et al., *Eur. J. Biochem.*, 1993, 215, 297-306; Fedoroff et al., *J. Mol. Biol.*, 1993, 233, 509-523; Gonzalez et al., *Biochemistry*, 1995, 34, 4969-4982; Horton et al., *J. Mol.*  
25 *Biol.*, 1996, 264, 521-533). The stability of a DNA:RNA hybrid is central to antisense therapies as the mechanism requires the binding of a modified DNA strand to a mRNA strand. To effectively inhibit the mRNA, the antisense DNA should have a very high binding affinity with the mRNA. Otherwise the desired interaction between the DNA and target mRNA strand will occur infrequently, thereby decreasing the efficacy of the antisense oligonucleotide.

30 Various synthetic modifications have been proposed to increase nuclease resistance, or to enhance the affinity of the antisense strand for its target mRNA (Crooke et al., *Med. Res. Rev.*, 1996, 16, 319-344; De Mesmaeker et al., *Acc. Chem. Res.*, 1995, 28, 366-374). A variety of modified phosphorus-containing linkages have been studied as replacements for the natural,

readily cleaved phosphodiester linkage in oligonucleotides. In general, most of them, such as the phosphorothioate, phosphoramidates, phosphonates and phosphorodithioates all result in oligonucleotides with reduced binding to complementary targets and decreased hybrid stability.

One synthetic 2'-modification that imparts increased nuclease resistance and a very high binding affinity to nucleotides is the 2'-methoxyethoxy (MOE, 2'-OCH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>) side chain (Baker et al., J. Biol. Chem., 1997, 272, 11944-12000; Freier et al., Nucleic Acids Res., 1997, 25, 4429-4443). One of the immediate advantages of the MOE substitution is the improvement in binding affinity, which is greater than many similar 2' modifications such as O-methyl, O-propyl, and O-aminopropyl (Freier and Altmann, Nucleic Acids Research, (1997) 25:4429-4443). 2'-O-methoxyethyl-substituted oligonucleotides also have been shown to be antisense inhibitors of gene expression with promising features for *in vivo* use (Martin, P., Helv. Chim. Acta, 1995, 78, 486-504; Altmann et al., Chimia, 1996, 50, 168-176; Altmann et al., Biochem. Soc. Trans., 1996, 24, 630-637; and Altmann et al., Nucleosides Nucleotides, 1997, 16, 917-926). Relative to DNA, they display improved RNA affinity and higher nuclease resistance. Chimeric oligonucleotides with 2'-O-methoxyethyl-ribonucleoside wings and a central DNA-phosphorothioate window also have been shown to effectively reduce the growth of tumors in animal models at low doses. MOE substituted oligonucleotides have shown outstanding promise as antisense agents in several disease states. One such MOE substituted oligonucleotide is presently being investigated in clinical trials for the treatment of CMV retinitis.

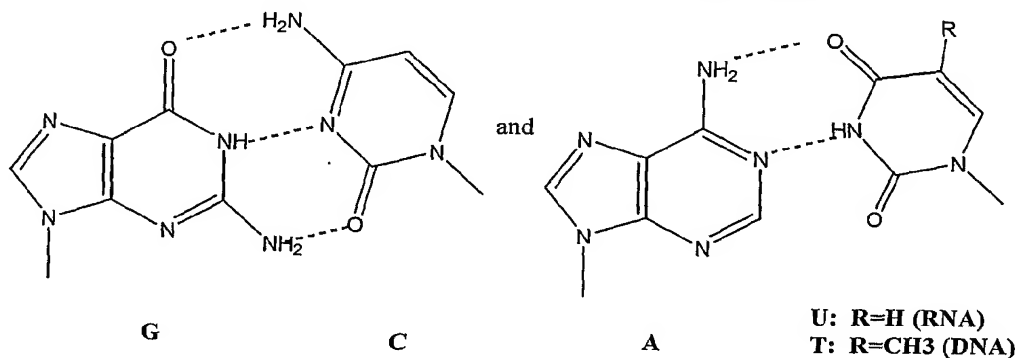
LNAs (oligonucleotides wherein the 2' and 4' positions are connected by a bridge) also form duplexes with complementary DNA, RNA or LNA with high thermal affinities. Circular dichroism (CD) spectra show that duplexes involving fully modified LNA (esp. LNA:RNA) structurally resemble an A-form RNA:RNA duplex. Nuclear magnetic resonance (NMR) examination of an LNA:DNA duplex confirmed the 3'-endo conformation of an LNA monomer. Recognition of double-stranded DNA has also been demonstrated suggesting strand invasion by LNA. Studies of mismatched sequences show that LNAs obey the Watson-Crick base pairing rules with generally improved selectivity compared to the corresponding unmodified reference strands. LNAs may be in either the  $\alpha$ -L- or the  $\beta$ -D- conformation. Vester et al., J.A.C.S., 124 (2002) 13682-13683.

LNAs in which the 2'-hydroxyl group is linked to the 4' carbon atom of the sugar ring thereby forming a 2'-C,4'-C-oxymethylene linkage thereby forming a bicyclic sugar moiety. The linkage can be an alkylene ((-CH<sub>2</sub>-)<sub>n</sub>) group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2 (Singh et al., Chem. Commun., 1998, 4, 455-456). LNA and LNA analogs

display very high duplex thermal stabilities with complementary DNA and RNA ( $T_m = +3$  to  $+10$  °C), stability towards 3'-exonucleolytic degradation and good solubility properties. Other suitable bridge groups include the 2'-CH<sub>2</sub>OCH<sub>2</sub>-4' bridge.

## 5 Nucleobases

The nucleobases Bx (also referred to in the art as nucleic acid bases or simply as bases) may be naturally-occurring G, C, A, U or T, or may be selected from a wide range of non-naturally occurring bases as described herein. The two most common classes of nucleobases are purines and pyrimidines. The naturally-occurring purine bases are guanine (G) and adenine (A), which are linked to the sugar through the 9-N nitrogen in the  $\beta$ -anomeric position on the sugar ring. The naturally-occurring pyrimidine bases are uracil (U), thymine (T) and cytidine (C), which are linked to the sugar through the 1-N nitrogen. In double stranded DNA (dsDNA), Watson-Crick base pairing occurs between G and C, and between A and T, whereas in double stranded RNA (dsRNA), Watson-Crick base pairing occurs between G and C, and between A and U. The Watson-Crick base pairs for DNA and RNA are shown below.



Analogous base pairing is generally observed in RNA-DNA hybrids, as well as in hybrids between naturally-occurring RNA or DNA and synthetic oligonucleotides comprising non-naturally occurring monomeric subunits.

20 In synthetic oligonucleotides according to the invention, such as antisense therapeutics and diagnostics, one or more of the naturally-occurring nucleobases may be replaced by an analogous binding member (nucleobase analog). Thus, the term “nucleobase” encompasses both naturally-occurring and non-naturally-occurring nucleobases. The term “nucleobase analog” (also referred to herein is a nucleobase mimetic or a nucleic acid base mimetic) refers to non-  
25 naturally-occurring nucleobases, and means a residue that functions like a nucleobase by providing sequence-specific binding to a heterocyclic residue on a complementary oligomer. In some embodiments according to the invention, a nucleobase analog is a residue that is capable of

establishing one or more non-covalent bonds with a nucleobase on a separate oligonucleotide strand. Non-covalent bonds are hydrogen bonds, ionic bonds and polar interactions. Additional interactions with non-complementary nucleobases are also possible, such as base-stacking interactions. In some embodiments of the invention, non-covalent bonds are formed by hydrogen bonding between nucleobase ring constituents and/or exocyclic substituents, and may be analogous to Watson-Crick bonding, Hoogsteen bonding, some combination thereof, or some other regime as described herein or as known in the art.

As used herein, "unmodified" or "natural" nucleobases mean the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases (nucleobase analogs) include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl ( $-C\equiv C-CH_3$ ) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine, 7-propynyl-7-deaza-8-azaguanine, 7-propynyl-7-deaza-8-azaadenine. Further modified nucleobases include, but are not limited to, tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido(5,4-b((1,4)benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido(5,4-b)(1,4)benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido(5,4-b)(1,4)benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido(4,5-b)indol-2-one), pyridoindole cytidine (H-pyrido(3',2':4,5)pyrrolo(2,3-d)pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include, but are not limited to, those disclosed in U.S. Patent No. 3,687,808, those disclosed in The Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., Angewandte Chemie, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Chapter 15, Antisense Research and Applications, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993.

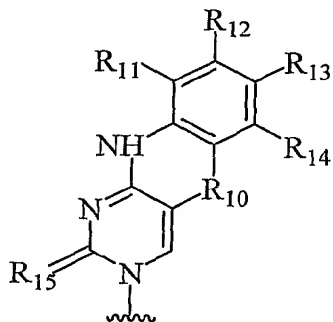


Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil, and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., Antisense Research and Applications, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently suitable base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative U.S. patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, 3,687,808; 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; 5,681,941; and 5,750,692.

In general, the term "base" includes the term nucleobase as described above. The term "base" means a binding member, as described hereinabove. While nucleobases are generally heterocyclic moieties, the term "base" as used herein with means any moiety or residue capable of participating in specific binding to a naturally-occurring nucleobase.

In some embodiments of the present invention oligomeric compounds are prepared having polycyclic heterocyclic compounds in place of one or more heterocyclic base moieties. A number of tricyclic heterocyclic compounds have been previously reported. These compounds are routinely used in antisense applications to increase the binding properties of the modified strand to a target strand. The most studied modifications selectively bind to guanosines. Hence they have been termed G-clamps or cytidine analogs. Many of these polycyclic heterocyclic compounds have the general formula:

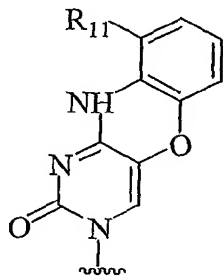


Representative cytosine analogs that make 3 hydrogen bonds with a guanosine in a second strand include 1,3-diazaphenoxazine-2-one ( $R_{10} = O$ ,  $R_{11} - R_{14} = H$ ) (Kurchavov, et al.,

Nucleosides and Nucleotides, 1997, 16, 1837-1846), 1,3-diazaphenothiazine-2-one ( $R_{10} = S$ ,  $R_{11} - R_{14} = H$ ), (Lin et al., J. Am. Chem. Soc., 1995, 117, 3873-3874) and 6,7,8,9-tetrafluoro-1,3-diazaphenoxazine-2-one ( $R_{10} = O$ ,  $R_{11} - R_{14} = F$ ) (Wang et al., Tetrahedron Lett., 1998, 39, 8385-8388). Incorporated into oligonucleotides these base modifications were shown to hybridize with complementary guanine and the latter was also shown to hybridize with adenine and to enhance helical thermal stability by extended stacking interactions (also see International Publication WO 02/097134, and U.S. Patent Application entitled "Nuclease Resistant Chimeric Oligonucleotides" filed May 24, 2002, U.S. Application Serial Number 10/013,295).

Further helix-stabilizing properties have been observed when a cytosine analog/substitute has an aminoethoxy moiety attached to the rigid 1,3-diazaphenoxazine-2-one scaffold ( $R_{10} = O$ ,  $R_{11} = -O-(CH_2)_2-NH_2$ ,  $R_{12-14} = H$ ) (Lin et al., J. Am. Chem. Soc., 1998, 120, 8531-8532). Binding studies demonstrated that a single incorporation could enhance the binding affinity of a model oligonucleotide to its complementary target DNA or RNA with a  $\Delta T_m$  of up to  $18^\circ$  relative to 5-methyl cytosine ( $dC5^{me}$ ), which is the highest known affinity enhancement for a single modification, yet. On the other hand, the gain in helical stability does not compromise the specificity of the oligonucleotides. The  $T_m$  data indicate an even greater discrimination between the perfect match and mismatched sequences compared to  $dC5^{me}$ . It was suggested that the tethered amino group serves as an additional hydrogen bond donor to interact with the Hoogsteen face, namely the O6, of a complementary guanine thereby forming 4 hydrogen bonds. This means that the increased affinity of G-clamp is mediated by the combination of extended base stacking and additional specific hydrogen bonding.

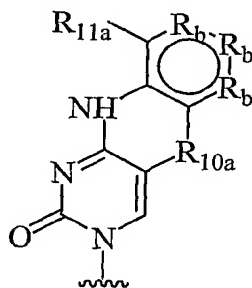
Further tricyclic heterocyclic compounds and methods of using them that are amenable to the present invention are disclosed in U.S. Patent Nos. 6,028,183 and 6,007,992. Such compounds include those having the formula:



wherein  $R_{11}$  includes  $(CH_3)_2N-(CH_2)_2-O-$ ;  $H_2N-(CH_2)_3-$ ;  $Ph-CH_2-O-C(=O)-N(H)-(CH_2)_3-$ ;  $H_2N-$ ; Fluorenyl- $CH_2-O-C(=O)-N(H)-(CH_2)_3-$ ; Phthalimidyl- $CH_2-O-C(=O)-N(H)-(CH_2)_3-$ ;  $Ph-CH_2-O-C(=O)-N(H)-(CH_2)_2-O-$ ;  $Ph-CH_2-O-C(=O)-N(H)-(CH_2)_3-O-$ ;  $(CH_3)_2N-N(H)-(CH_2)_2-O-$ ;

Fluorenyl-CH<sub>2</sub>-O-C(=O)-N(H)-(CH<sub>2</sub>)<sub>2</sub>-O-; Fluorenyl-CH<sub>2</sub>-O-C(=O)-N(H)-(CH<sub>2</sub>)<sub>3</sub>-O-; H<sub>2</sub>N-(CH<sub>2</sub>)<sub>2</sub>-O-CH<sub>2</sub>-; N<sub>3</sub>-(CH<sub>2</sub>)<sub>2</sub>-O-CH<sub>2</sub>-; H<sub>2</sub>N-(CH<sub>2</sub>)<sub>2</sub>-O-, and NH<sub>2</sub>C(=NH)NH-.

Also disclosed are tricyclic heterocyclic compounds of the formula:



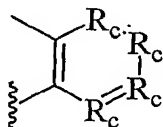
10 wherein:

R<sub>10a</sub> is O, S or N-CH<sub>3</sub>;

R<sub>11a</sub> is A(Z)<sub>X1</sub>, wherein A is a spacer and Z independently is a label bonding group optionally bonded to a detectable label, but R<sub>11a</sub> is not amine, protected amine, nitro or cyano;

15 X<sub>1</sub> is 1, 2 or 3; and

R<sub>b</sub> is independently -CH=, -N=, -C(C<sub>1-8</sub> alkyl)= or -C(halogen)=, but no adjacent R<sub>b</sub> are both -N=, or two adjacent R<sub>b</sub> are taken together to form a ring having the structure:

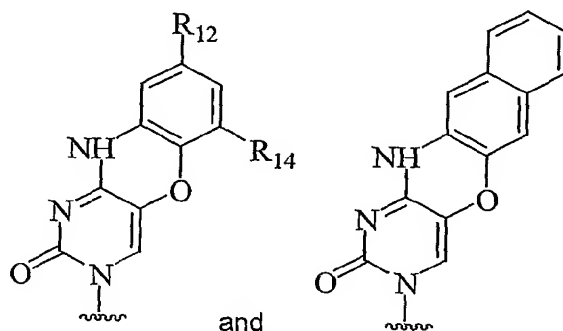


20 where R<sub>c</sub> is independently -CH=, -N=, -C(C<sub>1-8</sub> alkyl)= or -C(halogen)=, but no adjacent R<sub>b</sub> are both -N=.

The enhanced binding affinity of the phenoxazine derivatives together with their uncompromised sequence specificity makes them valuable nucleobase analogs for the development of more potent antisense-based drugs. In fact, promising data have been derived from *in vitro* experiments demonstrating that heptanucleotides containing phenoxazine substitutions are capable to activate RNaseH, enhance cellular uptake and exhibit an increased antisense activity (Lin et al., J. Am. Chem. Soc., 1998, 120, 8531-8532). The activity enhancement was even more pronounced in case of G-clamp, as a single substitution was shown to significantly improve the *in vitro* potency of a 20-mer 2'-deoxyphosphorothioate oligonucleotides (Flanagan et al., Proc. Natl. Acad. Sci. USA, 1999, 96, 3513-3518). Nevertheless, to optimize oligonucleotide design and to better understand the impact of these

heterocyclic modifications on the biological activity, it is important to evaluate their effect on the nuclease stability of the oligomers.

Further tricyclic and tetracyclic heteroaryl compounds amenable to the present invention include those having the formulas:

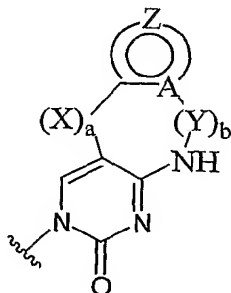


wherein:

$R_{14}$  is  $\text{NO}_2$  or both  $R_{14}$  and  $R_{12}$  are independently  $-\text{CH}_3$ .

The synthesis of these compounds is disclosed in, for example, U.S. Patent Nos. 5,434,257, 5,502,177, and 5,646, 269. Additional tricyclic heterocyclic compounds amenable to the present invention also disclosed in these patents include those having the formula:

10



a and b are independently 0 or 1 with the total of a and b being 0 or 1;

A is N, C or CH;

X is S, O, C=O, NH or  $\text{NCH}_2$ ,  $\text{R}^6$ ;

Y is C=O;

15

Z is taken together with A to form an aryl or heteroaryl ring structure comprising 5 or 6 ring atoms wherein the heteroaryl ring comprises a single O ring heteroatom, a single N ring heteroatom, a single S ring heteroatom, a single O and a single N ring heteroatom separated by a carbon atom, a single S and a single N ring heteroatom separated by a C atom, 2 N ring heteroatoms separated by a carbon atom, or 3 N ring heteroatoms at least 2 of which are separated by a carbon atom, and wherein the aryl or heteroaryl ring carbon atoms are

20

unsubstituted with other than H or at least 1 non-bridging ring carbon atom is substituted with  $R^{20}$  or  $=O$ ;

or Z is taken together with A to form an aryl ring structure comprising 6 ring atoms wherein the aryl ring carbon atoms are unsubstituted with other than H or at least 1 non-bridging  
5 ring carbon atom is substituted with  $R^6$  or  $=O$ ;

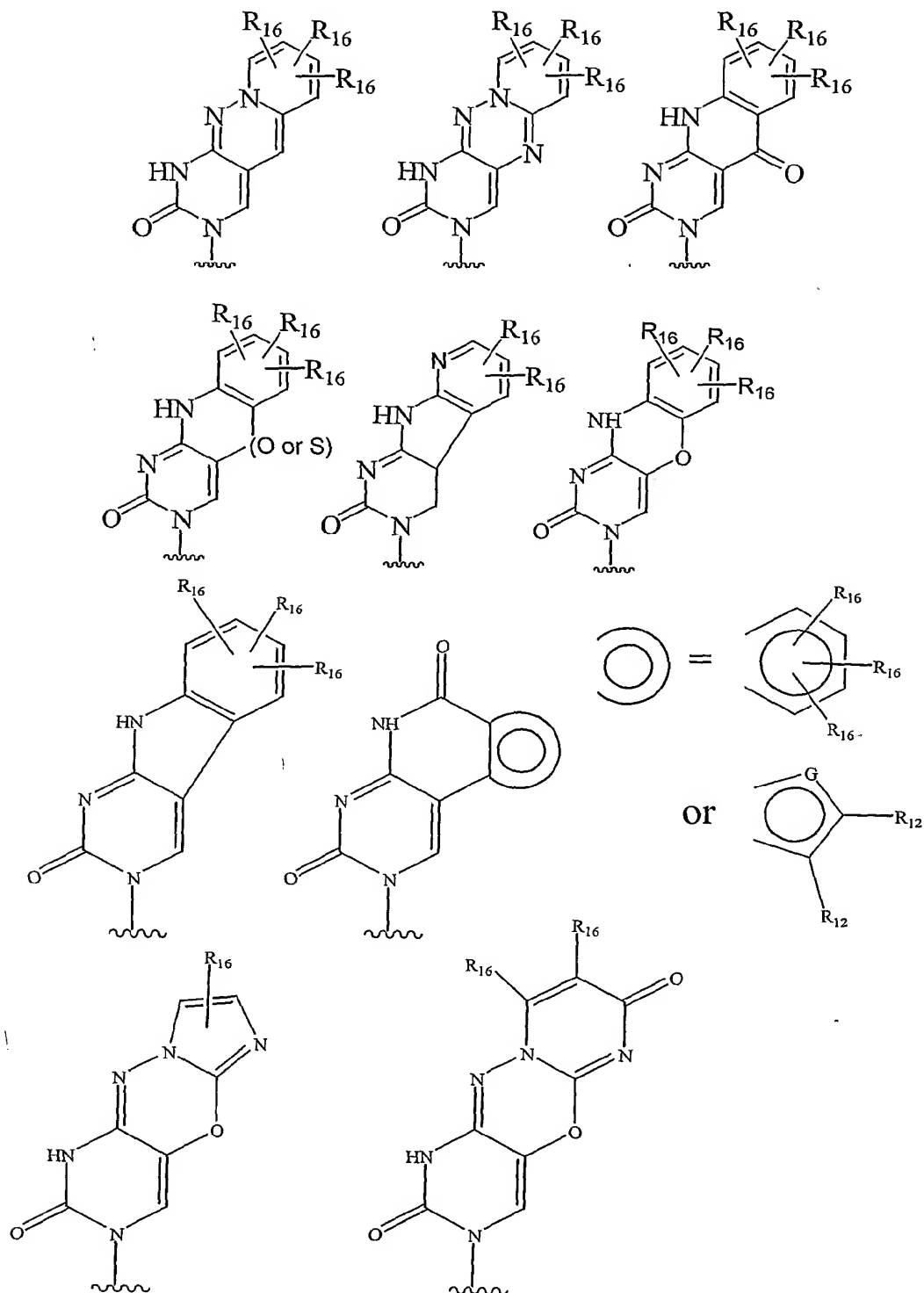
$R^6$  is, independently, H,  $C_{1-6}$  alkyl,  $C_{2-6}$  alkenyl,  $C_{2-6}$  alkynyl,  $NO_2$ ,  $N(R^3)_2$ , CN or halo, or an  $R^6$  is taken together with an adjacent Z group  $R^6$  to complete a phenyl ring;

$R^{20}$  is, independently, H,  $C_{1-6}$  alkyl,  $C_{2-6}$  alkyl,  $C_{2-6}$  alkenyl,  $C_{2-6}$  alkynyl,  $NO_2$ ,  $N(R^{21})_2$ , CN, or halo, or an  $R^{20}$  is taken together with an adjacent  $R^{20}$  to complete a ring containing 5 or 6  
10 ring atoms, and tautomers, solvates and salts thereof;

$R^{21}$  is, independently, H or a protecting group;

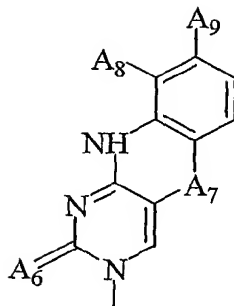
$R^3$  is a protecting group or H; and tautomers, solvates and salts thereof.

More specific examples included in the "257, 177 and 269" Patents are compounds of the formula:



wherein each  $R_{16}$ , is, independently, selected from hydrogen and various substituent groups.

Further polycyclic base moieties having the formula:

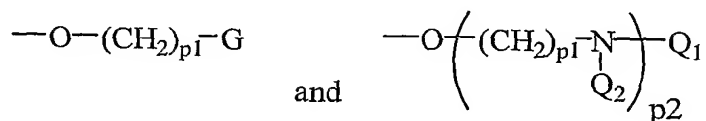


wherein:

A<sub>6</sub> is O or S;

A<sub>7</sub> is CH<sub>2</sub>, N-CH<sub>3</sub>, O or S;

- 5 each A<sub>8</sub> and A<sub>9</sub> is hydrogen or one of A<sub>8</sub> and A<sub>9</sub> is hydrogen and the other of A<sub>8</sub> and A<sub>9</sub> is selected from the group consisting of:



wherein:

G is -CN, -OA<sub>10</sub>, -SA<sub>10</sub>, -N(H)A<sub>10</sub>, -ON(H)A<sub>10</sub> or -C(=NH)N(H)A<sub>10</sub>;

- 10 Q<sub>1</sub> is H, -NHA<sub>10</sub>, -C(=O)N(H)A<sub>10</sub>, -C(=S)N(H)A<sub>10</sub> or -C(=NH)N(H)A<sub>10</sub>;

each Q<sub>2</sub> is, independently, H or Pg;

A<sub>10</sub> is H, Pg, substituted or unsubstituted C<sub>1</sub>-C<sub>10</sub> alkyl, acetyl, benzyl, -(CH<sub>2</sub>)<sub>p3</sub>NH<sub>2</sub>, -(CH<sub>2</sub>)<sub>p3</sub>N(H)Pg, a D or L α-amino acid, or a peptide derived from D, L or racemic α-amino acids;

- 15 Pg is a nitrogen, oxygen or thiol protecting group;

each p<sub>1</sub> is, independently, from 2 to about 6;

p<sub>2</sub> is from 1 to about 3; and

p<sub>3</sub> is from 1 to about 4;

are disclosed in International Publication WO 03/004602.

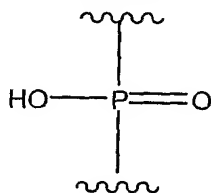
- 20 In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a
- 25 nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a

nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can 5 hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable.

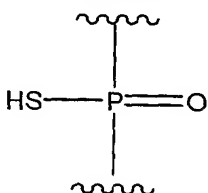
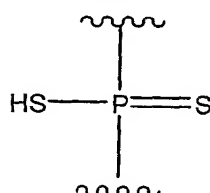
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### *Phosphate Linkers*

Oligonucleotides are generally those oligomers in which the monomeric subunits comprise linking members having pentavalent phosphorus as a constituent part. Phosphate linkers include phosphodiester, phosphorothioate and phosphorodithioate linkers.



phosphodiester

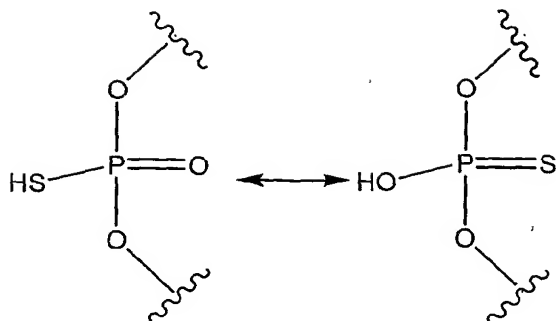
phosphorothioate  
diesterphosphorodithioate  
diester

15

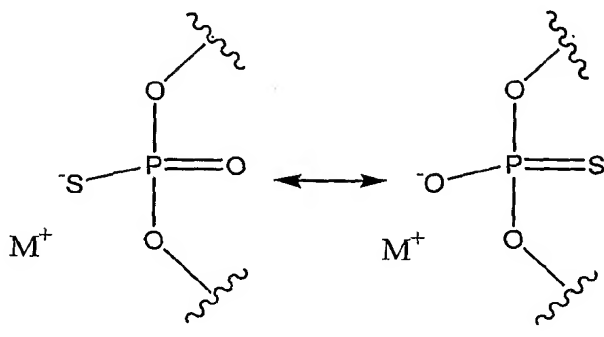
The squiggles (~) indicate covalent bonds to backbone members, e.g. oxygen atoms on sugar backbone moieties, or other substituent on sugar analogs.

Oligonucleotides as defined herein generally include salts, solvates and tautomers of oligonucleotides. In general, many bases, especially nucleobases, can form tautomeric structures 20 that are included within the general definitions of oligonucleotides according to the present invention. In addition, the phosphorothioate linker can form the following tautomers:





and can likewise form the following salt structures:



wherein  $M^+$  is a suitable salt-forming cation, such as  $Na^+$ ,  $K^+$ ,  $\frac{1}{2} Ca^{2+}$ ,  $\frac{1}{2} Mg^{2+}$ ,  $\frac{1}{3} Al^{3+}$ ,  $NH_4^+$ ,  $H_3O^+$ , and the like. The fractions indicate fractional equivalents of the cationic species per phosphate diester linkage. Phosphodiester and phosphorodithioate moieties can form analogous salts.

Naturally occurring nucleosides are linked to one another via a phosphodiester linker. Antisense compounds may be prepared using phosphodiester linkers, which are generally suitable for diagnostic and other nuclease-free uses. However, antisense therapeutic compounds advantageously comprise at least one phosphorothioate linker, owing to the latter's superior nuclease stability. Both phosphodiester and phosphorothioate diester linkers are generally referred to as phosphate diester linkers. When a plurality of nucleotides are linked by successive phosphate diester linkers, the resulting oligomer is called an oligonucleotide.

#### *Manufacture of Oligonucleotides*

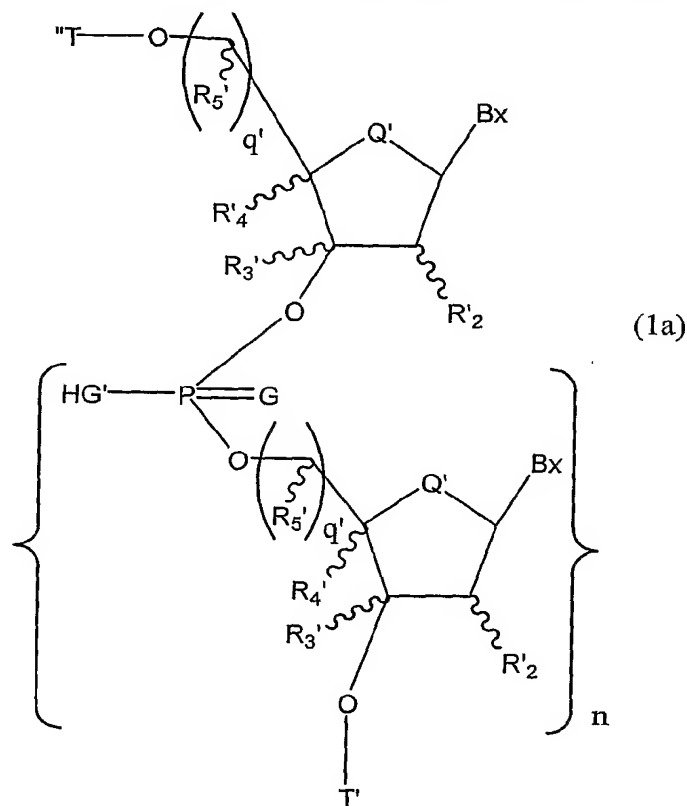
As described above, the term "oligonucleotide" encompasses naturally-occurring RNA and DNA as well as phosphate-linked oligomers having a variety of sugar backbones and nucleobases. Oligonucleotides have been made by the phosphate triester, H-phosphonate and phosphoramidite methods as described hereinabove. Of these three methods, the phosphoramidite method has become the de facto standard for oligonucleotide synthesis,

especially where one or more modifications are made to the sugar backbone or nucleobases, or where exceptional purity, yield or scale are paramount. The phosphoramidite method (amidite method) is described hereinafter.

### 5 Amidite Method

Oligonucleotides according to embodiments of the present invention are represented by formula 1, above.

While the present invention is concerned primarily with oligonucleotides, some oligonucleotide mimetics may, with appropriate changes to the starting materials, also be prepared by processes according to the present invention. Oligonucleotide mimetics include compounds in which the oligonucleotide sugar has been replaced with a heterocyclic or carbocyclic ring structure. Such compounds are depicted in Formula 1a, below.



and tautomers, salts and solvates thereof, wherein G, G', Bx, n, R<sub>2</sub>', R<sub>3</sub>', R<sub>4</sub>' and R<sub>5</sub>' each have the meanings previously defined. The groups T' and T'' are each H, or conjugate groups, such as protecting groups and substituents. Each Q' is independently O, S, NR''', C(R''')<sub>2</sub>, or -CR'''=CR'''-, where each R''' is H, alkyl, or where two R''' groups are on the same or adjacent carbon atoms,

they may form a carbocyclic or heterocyclic ring, wherein the ring contains one or two of N, O or S. Suitable values of R<sup>m</sup> are H and C<sub>1</sub>-C<sub>4</sub> alkyl.

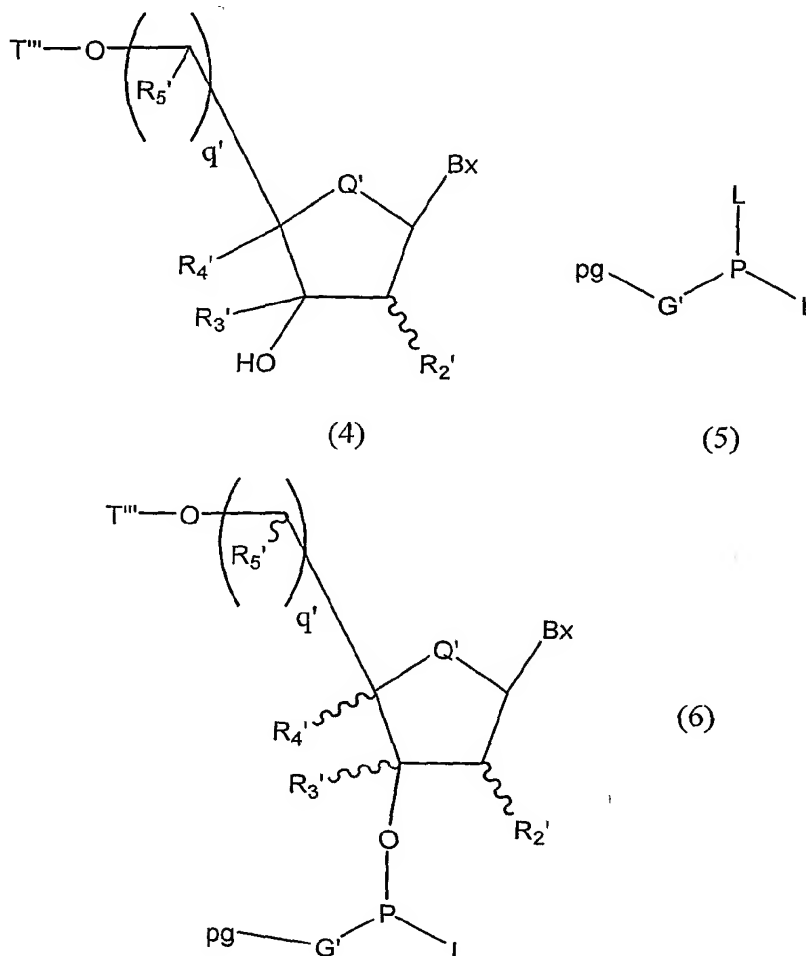
The foregoing oligonucleotides and oligonucleotide mimetics may be manufactured by solid phase synthesis, e.g. by the amidite method. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Other means for such synthesis known in the art may additionally or alternatively be employed. For example stirred-bed reactors have been used.

Support bound oligonucleotide synthesis relies on sequential addition of nucleotides to one end of a growing chain. Typically, a first nucleoside (having protecting groups on any exocyclic amine functionalities present) is attached to an appropriate glass bead support and activated phosphite compounds (typically nucleotide phosphoramidites, also bearing appropriate protecting groups) are added stepwise to elongate the growing oligonucleotide. Additional methods for solid-phase synthesis may be found in U.S. Patents Nos. 4,415,732; 4,458,066; 4,500,707; 4,668,777; 4,973,679; and 5,132,418; and 4,725,677, and Re. 34,069.

Examples of the synthesis of particular modified oligonucleotides may be found in the following U.S. patents or pending patent applications, each of which is commonly assigned with this application: 5,138,045 and 5,218,105, drawn to polyamine conjugated oligonucleotides; 5,212,295, drawn to monomers for the preparation of oligonucleotides having chiral phosphorus linkages; 5,378,825 and 5,541,307, drawn to oligonucleotides having modified backbones; 5,386,023, drawn to backbone modified oligonucleotides and the preparation thereof through reductive coupling; 5,457,191, drawn to modified nucleobases based on the 3-deazapurine ring system and methods of synthesis thereof; 5,459,255, drawn to modified nucleobases based on N-2 substituted purines; 5,521,302, drawn to processes for preparing oligonucleotides having chiral phosphorus linkages; 5,539,082, drawn to peptide nucleic acids; 5,554,746, drawn to oligonucleotides having  $\beta$ -lactam backbones; 5,571,902, drawn to methods and materials for the synthesis of oligonucleotides; 5,578,718, drawn to nucleosides having alkylthio groups, wherein such groups may be used as linkers to other moieties attached at any of a variety of positions of the nucleoside; 5,587,361 and 5,599,797, drawn to oligonucleotides having phosphorothioate linkages of high chiral purity; 5,506,351, drawn to processes for the preparation of 2'-O-alkyl guanosine and related compounds, including 2,6-diaminopurine compounds; 5,587,469, drawn to oligonucleotides having N-2 substituted purines; 5,587,470, drawn to oligonucleotides having 3-deazapurines; 5,223,168, and 5,608,046, both drawn to conjugated 4'-desmethyl nucleoside analogs; 5,602,240, and 5,610,289, drawn to backbone modified oligonucleotide analogs; and

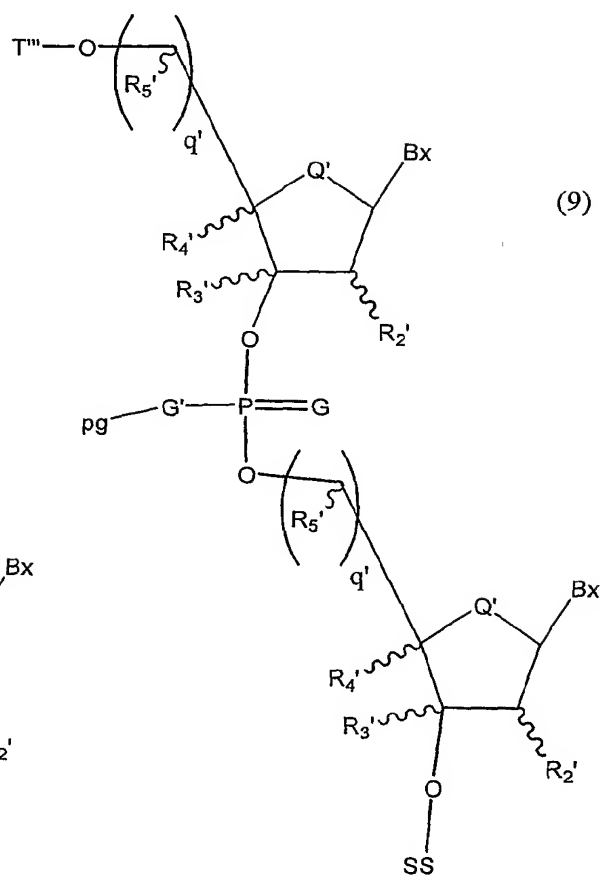
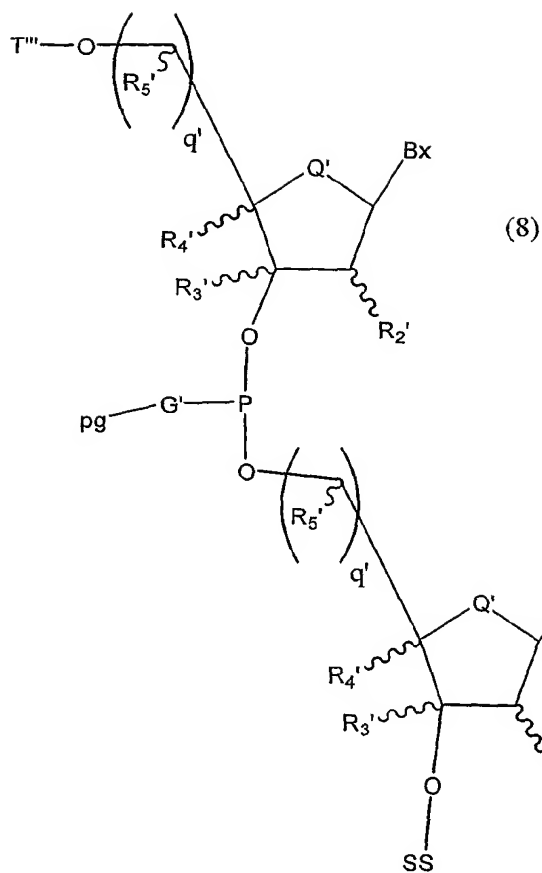
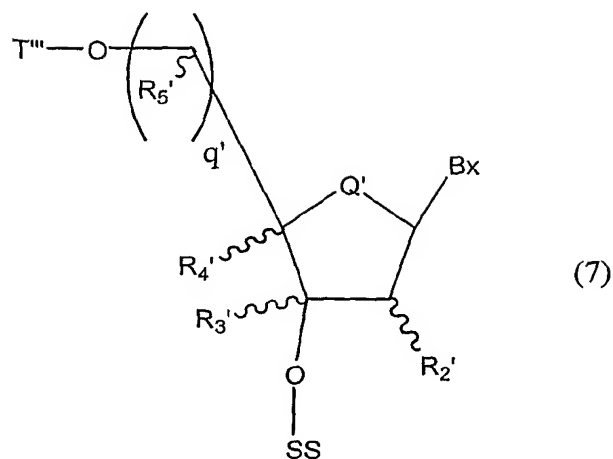
U.S. Patent Application Serial No. 08/383,666, filed February 3, 1995, and U.S. Patent No. 5,459,255, drawn to, inter alia, methods of synthesizing 2'-fluoro-oligonucleotides.

The amidite method of oligonucleotide synthesis may be carried out generally in the following manner: Phosphoramidites are prepared by reacting a suitable nucleoside or modified  
5 nucleoside (formula 4) with a phosphorodiamidite (formula 5) to form a phosphoramidite (formula 6).

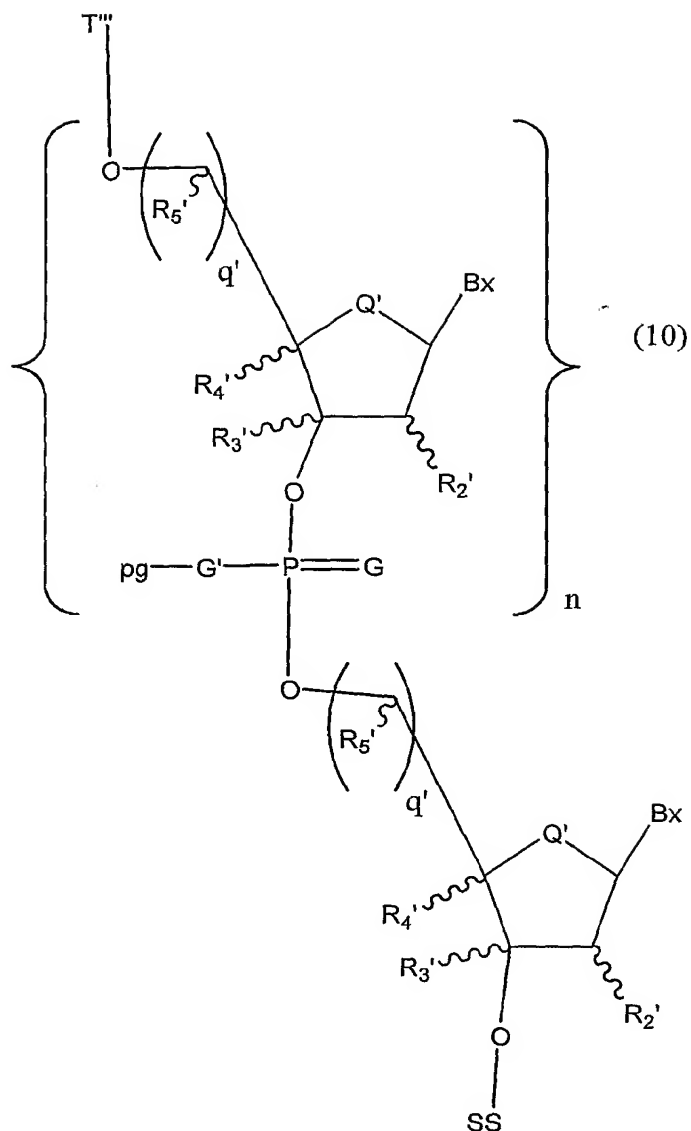


Each of the variables Q', Bx, R<sub>2</sub>', R<sub>3</sub>', R<sub>4</sub>', R<sub>5</sub>', G', and q' is as previously defined. L is an amine  
10 leaving group; pg is a phosphorus protecting group; and T''' is a hydroxyl protecting group, each as more specifically defined herein.

A support-bound nucleoside of Formula 7 is first deprotected at the 5'-position  
(resulting in a free 5'-OH group), after which a first amidite is coupled to a support-bound  
nucleoside to form a support-bound dimer of Formula 8, which is then oxidized, and subjected to  
15 a capping step to form a support bound dimer of Formula 9.



The 5'-deprotection, coupling, oxidation and capping steps are then repeated  $n-2$  times to form a support-bound oligomer of Formula 10.



This compound (10) is then cleaved from the solid support, 5'-deprotected, and purified to yield an oligomer of Formula (1). The oligonucleotide may then be further derivatized, purified, precipitated, or otherwise treated, as described in more detail herein.

- 5 In each of the foregoing Formulae, SS represents a support bound to the 3'-terminal nucleoside by a cleavable linker, each pg is a phosphorus protecting group as defined herein, n is an integer, G and G' are independently O or S, and each Bx, R<sub>2</sub>', R<sub>3</sub>', R<sub>4</sub>', R<sub>5</sub>', Q', and q' is independently as defined in Formula 3.

*Amidites*

Phosphoramidites (amidites) used in the synthesis of oligonucleotides are available from a variety of commercial sources (included are: Glen Research, Sterling, Virginia; Amersham Pharmacia Biotech Inc., Piscataway, New Jersey; Cruachem Inc., Aston, Pennsylvania; Chemgenes Corporation, Waltham, Massachusetts; Proligo LLC, Boulder, Colorado; PE Biosystems, Foster City California; Beckman Coulter Inc., Fullerton, California). These commercial sources sell high purity phosphoramidites generally having a purity of better than 98%. Those not offering an across the board purity for all amidites sold will in most cases include an assay with each lot purchased giving at least the purity of the particular phosphoramidite purchased. Commercially available phosphoramidites are prepared for the most part for automated DNA synthesis and as such are prepared for immediate use for synthesizing desired sequences of oligonucleotides. Phosphoramidites may be prepared by methods disclosed in, for example, U.S. Patent Nos. 4,415,732; 4,458,066; 4,500,707; 4,668,777; 4,973,679; and 5,132,418 and U.S. RE 34,069).

*Support Media*

Oligonucleotides are generally prepared, as described above, on a support medium (support), e.g. a solid support medium. In general a first synthon (e.g. a monomer, such as a nucleoside) is first attached to a support medium, and the oligonucleotide is then synthesized by sequentially coupling monomers to the support-bound synthon. This iterative elongation eventually results in a final oligomeric compound or other polymer such as a polypeptide. Suitable support media can be soluble or insoluble, or may possess variable solubility in different solvents to allow the growing support bound polymer to be either in or out of solution as desired. Traditional support media such as solid supports are for the most part insoluble and are routinely placed in reaction vessels while reagents and solvents react with and/or wash the growing chain until the oligomer has reached the target length, after which it is cleaved from the support and, if necessary further worked up to produce the final polymeric compound. More recent approaches have introduced soluble supports including soluble polymer supports to allow precipitating and dissolving the iteratively synthesized product at desired points in the synthesis (Gravert et al., Chem. Rev., 1997, 97, 489-510).

The term support media (support) is intended to include supports known to the person skilled in the art to for the synthesis of oligomeric compounds and related compounds such as peptides. Some representative support media that are amenable to the methods of the present

invention include, but are not limited to, the following: controlled pore glass (CPG); oxalyl-controlled pore glass (see, e.g., Alul, et al., *Nucleic Acids Research* 1991, 19, 1527); silica-containing particles, such as porous glass beads and silica gel such as that formed by the reaction of trichloro-(3-(4-chloromethyl)phenyl)propylsilane and porous glass beads (see Parr and  
5 Grohmann, *Angew. Chem. Internal. Ed.*, 1972, 11, 314, sold under the trademark "PORASIL E" by Waters Associates, Framingham, Mass., USA); the mono ester of 1,4-dihydroxymethylbenzene and silica (see Bayer and Jung, *Tetrahedron Lett.*, 1970, 4503, sold under the trademark "BIOPAK" by Waters Associates); TENTAGEL (see, e.g., Wright et al., *Tetrahedron Letters* 1993, 34, 3373); cross-linked styrene/divinylbenzene copolymer beaded  
10 matrix or POROS, a copolymer of polystyrene/divinylbenzene (available from Perceptive Biosystems); soluble support media, polyethylene glycol PEG's (see Bonora et al., *Organic Process Research & Development*, 2000, 4, 225-231).

Further support media amenable to the present invention include without limitation PEPS support a polyethylene (PE) film with pendant long-chain polystyrene (PS) grafts  
15 (molecular weight on the order of  $10^6$ , (see Berg et al., *J. Am. Chem. Soc.*, 1989, 111, 8024 and International Patent Application WO 90/02749)). The loading capacity of the film is as high as that of a beaded matrix with the additional flexibility to accommodate multiple syntheses simultaneously. The PEPS film may be fashioned in the form of discrete, labeled sheets, each serving as an individual compartment. During all the identical steps of the synthetic cycles, the  
20 sheets are kept together in a single reaction vessel to permit concurrent preparation of a multitude of peptides at a rate close to that of a single peptide by conventional methods. Also, experiments with other geometries of the PEPS polymer such as, for example, non-woven felt, knitted net, sticks or microwell plates have not indicated any limitations of the synthetic efficacy.

Further support media amenable to the present invention include without limitation  
25 particles based upon copolymers of dimethylacrylamide cross-linked with *N,N'*-bisacryloylethylenediamine, including a known amount of *N*-tert-butoxycarbonyl-beta-alanyl-*N'*-acryloylhexamethylenediamine. Several spacer molecules are typically added via the beta alanyl group, followed thereafter by the amino acid residue subunits. Also, the beta alanyl-containing monomer can be replaced with an acryloyl safcosine monomer during polymerization to form  
30 resin beads. The polymerization is followed by reaction of the beads with ethylenediamine to form resin particles that contain primary amines as the covalently linked functionality. The polyacrylamide-based supports are relatively more hydrophilic than are the polystyrene-based supports and are usually used with polar aprotic solvents including dimethylformamide,



dimethylacetamide, N-methylpyrrolidone and the like (see Atherton, et al., J. Am. Chem. Soc., 1975, 97, 6584, Bioorg. Chem. 1979, 8, 351, and J. C. S. Perkin I 538 (1981)).

Other support media amenable to the present invention include without limitation a composite of a resin and another material that is also substantially inert to the organic synthesis reaction conditions employed. One exemplary composite (see Scott, et al., J. Chrom. Sci., 1971, 9, 577) utilizes glass particles coated with a hydrophobic, cross-linked styrene polymer containing reactive chloromethyl groups, and is supplied by Northgate Laboratories, Inc., of Hamden, Conn., USA. Another exemplary composite contains a core of fluorinated ethylene polymer onto which has been grafted polystyrene (see Kent and Merrifield, Israel J. Chem. 1978, 17, 243 and van Rietschoten in Peptides 1974, Y. Wolman, Ed., Wiley and Sons, New York, 1975, pp. 113-116). Contiguous solid supports other than PEPS, such as cotton sheets (Lebl and Eichler, Peptide Res. 1989, 2, 232) and hydroxypropylacrylate-coated polypropylene membranes (Daniels, et al., Tetrahedron Lett. 1989, 4345). Acrylic acid-grafted polyethylene-rods and 96-microtiter wells to immobilize the growing peptide chains and to perform the compartmentalized synthesis. (Geysen, et al., Proc. Natl. Acad. Sci. USA, 1984, 81, 3998). A "tea bag" containing traditionally-used polymer beads. (Houghten, Proc. Natl. Acad. Sci. USA, 1985, 82, 5131). Simultaneous use of two different supports with different densities (Tregear, Chemistry and Biology of Peptides, J. Meienhofer, ed., Ann Arbor Sci. Publ., Ann Arbor, 1972 pp. 175-178). Combining of reaction vessels via a manifold (Gorman, Anal. Biochem., 1984, 136, 397). Multicolumn solid-phase synthesis (e.g., Krehnak, et al., Int. J. Peptide Protein Res., 1989, 33, 209), and Holm and Meldal, in "Proceedings of the 20th European Peptide Symposium", G. Jung and E. Bayer, eds., Walter de Gruyter & Co., Berlin, 1989 pp. 208-210). Cellulose paper (Eichler, et al., Collect. Czech. Chem. Commun., 1989, 54, 1746). Support mediated synthesis of peptides have also been reported (see, Synthetic Peptides: A User's Guide, Gregory A. Grant, Ed. Oxford University Press 1992; U.S. Patent Nos. 4,415,732; 4,458,066; 4,500,707; 4,668,777; 4,973,679; 5,132,418; 4,725,677 and Re-34,069.)

### *Equipment for Synthesis*

Commercially available equipment routinely used for the support media based synthesis of oligomeric compounds and related compounds is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. Suitable solid phase techniques, including

automated synthesis techniques, are described in F. Eckstein (ed.), *Oligonucleotides and Analogues, a Practical Approach*, Oxford University Press, New York (1991).

### *Phosphorus Protecting Groups*

5 In general, the phosphorus protecting group (pg) is an alkyl group or a  $\beta$ -eliminable group having the formula  $-\text{CH}_2\text{CH}_2-\text{G}_w$ , wherein  $\text{G}_w$  is an electron-withdrawing group. Suitable examples of pg that are amenable to use in connection with the present invention include those set forth in U.S. Patents Nos. 4,415,732; 4,458,066; 4,500,707; 4,668,777; 4,973,679; and 5,132,418; and U.S. Patents Nos. 4,725,677 and Re. 34,069. In general the alkyl or cyanoethyl  
10 withdrawing groups are suitable, as commercially available phosphoramidites generally incorporate either the methyl or cyanoethyl phosphorus protecting group.

The method for removal of phosphorus protecting groups (pg's) depends upon the specific pg to be removed. The  $\beta$ -eliminable groups, such as those disclosed in the Köster et al. patents, are generally removed in a weak base solution, whereby an acidic  $\beta$ -hydrogen is  
15 extracted and the  $-\text{CH}_2\text{CH}_2-\text{G}_w$  group is eliminated by rearrangement to form the corresponding acrylo-compound  $\text{CH}_2=\text{CH}-\text{G}_w$ . In contrast, an alkyl group is generally removed by nucleophilic attack on the  $\alpha$ -carbon of the alkyl group. Such pg's are described in the Caruthers et al. patents, as cited herein.

### 20 *Oxidation (Including Sulfurization)*

The person skilled in the art will recognize that oxidation of P(III) to P(V) can be carried out by a variety of reagents. Furthermore, the person skilled in the art will recognize that the P(V) species can exist as phosphate triesters, phosphorothioate diesters, or phosphorodithioate diesters. Each type of P(V) linkage has uses and advantages, as described  
25 herein. Thus, the term "oxidizing agent" should be understood broadly as being any reagent capable of transforming a P(III) species (e.g. a phosphite) into a P(V) species. Thus, the term "oxidizing agent" includes "sulfurizing agent," and oxidation will be understood to embrace both introduction of oxygen and introduction of sulfur, or sulfurization. Where it is important to indicate that an oxidizing agent introduces an oxygen into a P(III) species to make a P(V)  
30 species, the oxidizing agent will be referred to herein is "an oxygen-introducing oxidizing reagent."

Oxidizing reagents for making phosphate diester linkages (i.e. oxygen-introducing oxidizing reagents) under the phosphoramidite protocol have been described by e.g. Caruthers et

al. and Köster et al., as cited herein. Examples of sulfurization reagents which have been used to synthesize oligonucleotides containing phosphorothioate bonds include elemental sulfur, dibenzoyltetrasulfide, 3-H-1,2-benzidithiol-3-one 1,1-dioxide (also known as Beaucage reagent), tetraethylthiuram disulfide (TETD), and bis-(O,O-diisopropoxy phosphinothioyl) disulfide  
5 (known as Stec reagent). Oxidizing reagents for making phosphorothioate diester linkages include phenyl acetyl disulfide (PADS), as described in U.S. Patent No. 6,242,591. In some embodiments of the invention, the phosphorothioate diester and phosphate diester linkages may alternate between sugar subunits. In other embodiments of the present invention, phosphorothioate linkages alone may be employed.

10 Various solvents may be used in the oxidation reaction. Suitable solvents are identified in the Caruthers et al. and Köster et al. patents, cited herein. The Cole et al. patent describes acetonitrile as a solvent for phenyl acetyl disulfide. Other suitable solvents include, but are not limited to, toluene, xanthenes, dichloromethane, and the like.

### 15 *Cleavage and Workup*

Reagents for cleaving an oligonucleotide from a support are set forth, for example, in the Caruthers et al. and Köster et al. patents, as cited herein.

The oligonucleotide may be worked up by standard procedures known in the art, for example by size exclusion chromatography, high performance liquid chromatography (e.g.  
20 reverse-phase HPLC), differential precipitation, etc. In some embodiments according to the present invention, the oligonucleotide is cleaved from a solid support while the 5'-OH protecting group is still on the ultimate nucleoside. This so-called DMT-on (or trityl-on) oligonucleotide is then subjected to chromatography, after which the DMT group is removed by treatment in an organic acid, after which the oligonucleotide is de-salted and further purified to form a final  
25 product.

### *5'-Deprotection*

The 5'-hydroxyl protecting groups may be any groups that are selectively removed under suitable conditions. In particular, the 4,4'-dimethoxytriphenylmethyl (DMT) group is a  
30 favored group for protecting at the 5'-position, because it is readily cleaved under acidic conditions (e.g. in the presence of dichloroacetic acid (DCA), trichloroacetic acid (TCA), or acetic acid. Removal of DMT from the support-bound oligonucleotide is generally performed

with DCA. Removal of 5'-protecting groups after cleavage of the oligonucleotide from the support is generally performed with acetic acid.

### *Oligonucleosides*

- 5 In addition to phosphate diester and phosphorothioate diester linkages, other linkers are known in the art. While the primary concern of the present invention is formation of oligomers having phosphate diester linkages, i.e. oligonucleotides, the present invention also broadly encompasses chimeric oligomers, i.e. oligomers having more than one type of linkage, backbone, or combination thereof. It is to be noted that most oligonucleotides have more than one type of
- 10 nucleobase, and thus oligomers having all phosphate diester linkages and sugar backbones are not, strictly speaking, chimeric merely because they have more than one type of nucleobase. A variety of non-phosphate linkages have been described in the art, and chimeric compounds containing both phosphate diester linkages and non-phosphate linkages are placed within the skill of the artisan by this disclosure.
- 15 An oligonucleoside is an oligomer having the same type of sugar backbone (optionally substituted as necessary to accommodate the alternative linking member) and nucleobase binding member as is generally found in oligonucleotides, however, the linking member differs in that it is not a phosphate linking member. Exemplary non-phosphate diester linkages contemplated within the skill of the art include, but are not limited to, phosphorodithioates, phosphotriesters,
- 20 aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates. Additional linkages include: thiodiester (-O-C(O)-S-),
- 25 thionocarbamate (-O-C(O)(NJ)-S-), siloxane (-O-Si(J)<sub>2</sub>-O-), carbamate (-O-C(O)-NH- and -NH-C(O)-O-), sulfamate (-O-S(O)(O)-N- and -N-S(O)(O)-N-, morpholino sulfamide (-O-S(O)(N(morpholino)-), sulfonamide (-O-SO<sub>2</sub>-NH-), sulfide (-CH<sub>2</sub>-S-CH<sub>2</sub>-), sulfonate (-O-SO<sub>2</sub>-CH<sub>2</sub>-), N,N'-dimethylhydrazine (-CH<sub>2</sub>-N(CH<sub>3</sub>)-N(CH<sub>3</sub>)-), thioformacetal (-S-CH<sub>2</sub>-O-), formacetal (-O-CH<sub>2</sub>-O-), thioketal (-S-C(J)<sub>2</sub>-O-), ketal (-O-C(J)<sub>2</sub>-O-), amine (-NH-CH<sub>2</sub>-CH<sub>2</sub>-),
- 30 hydroxylamine (-CH<sub>2</sub>-N(J)-O-), hydroxylimine (-CH=N-O-), and hydrazinyl (-CH<sub>2</sub>-N(H)-N(H)-).

In each of the foregoing substructures relating to internucleoside linkages, J denotes a substituent group which is commonly hydrogen or an alkyl group or a more complicated group that varies from one type of linkage to another.

In addition to linking groups as described above that involve the modification or substitution of the -O-P-O- atoms of a naturally occurring linkage, included within the scope of the present invention are linking groups that include modification of the 5'-methylene group as well as one or more of the -O-P-O- atoms. Linkages of this type are well documented in the prior art and include without limitation the following: amides (-CH<sub>2</sub>-CH<sub>2</sub>-N(H)-C(O)) and -CH<sub>2</sub>-O-N=CH-; and alkylphosphorus (-C(J)<sub>2</sub>-P(=O)(OJ)-C(J)<sub>2</sub>-C(J)<sub>2</sub>-). J is as described above.

Synthetic schemes for the synthesis of the substitute internucleoside linkages described above are disclosed in, for example, U.S. Patent Nos. 5,466,677; 5,034,506; 5,124,047; 5,278,302; 5,321,131; 5,519,126; 4,469,863; 5,455,233; 5,214,134; 5,470,967; 5,434,257. Additional background information relating to internucleoside linkages can be found in, for example, WO 91/08213; WO 90/15065; WO 91/15500; WO 92/20822; WO 92/20823; WO 91/15500; WO 89/12060; EP 216860; PCT/US 92/04294; PCT/US 90/03138; PCT/US 91/06855; PCT/US 92/03385; PCT/US 91/03680; U.S. Application Nos. 07/990,848; 07/892,902; 07/806,710; 07/763,130; 07/690,786; Stirchak et al., Nucleic Acid Res., 1989, 17, 6129-6141; Hewitt et al., 1992, 11, 1661-1666; Sood et al., J. Am. Chem. Soc., 1990, 112, 9000-9001; Vaseur et al., J. Amer. Chem. Soc., 1992, 114, 4006-4007; Musichi et al., J. Org. Chem., 1990, 55, 4231-4233; Reynolds et al., J. Org. Chem., 1992, 57, 2983-2985; Mertes et al., J. Med. Chem., 1969, 12, 154-157; Mungall et al., J. Org. Chem., 1977, 42, 703-706; Stirchak et al., J. Org. Chem., 1987, 52, 4202-4206; Coull et al., Tet. Lett., 1987, 28, 745; and Wang et al., Tet. Lett., 1991, 32, 7385-7388.

## Other Monomeric Subunits

Synthetic oligomers have been described which use a backbone other than a sugar or modified sugar, a linker other than a phosphate linker, or both. Chimeric compounds have been developed that combine at least one stretch of oligonucleotide sequence with at least one stretch of an oligomer other than oligonucleotide, e.g. an oligonucleoside, PNA, morpholino, or other type of monomeric subunit.

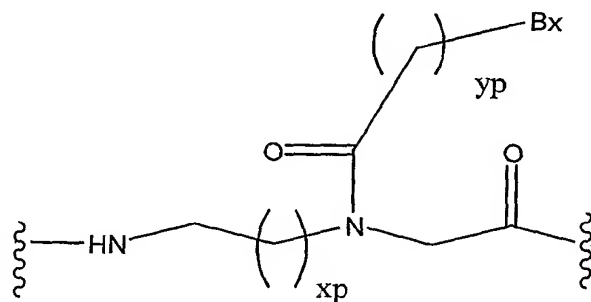
In some embodiments according to the present invention, the term "oligonucleotide mimetic" refers to an oligonucleotide having backbone members other than sugar groups. Although the term is intended to include oligomeric compounds wherein only the furanose ring

or both the furanose ring and the internucleotide linkage are replaced with novel groups, replacement of only the furanose ring is also referred to in the art as being a sugar surrogate. Oligonucleotide mimetics can be further modified to incorporate one or more modified heterocyclic base moieties to enhance properties such as hybridization.

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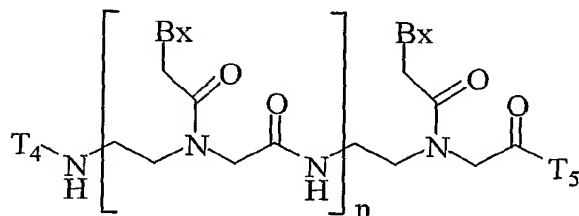
### *Peptide Nucleic Acids*

One oligonucleotide mimetic that has been reported to have excellent hybridization properties, is peptide nucleic acids (PNA). PNAs (peptide nucleic acids) use the uncharged amide bond instead of a phosphate diester bond to link adjacent sugar moieties. In the case of  
10 PNAs, the linker may perform the linking function as well as the functions performed by the sugar moiety in naturally occurring oligonucleotides. A typical PNA subunit is depicted below, wherein xp and yp are each 0 or an integer of about 1 to about 5, especially about 1, and Bx is a nucleobase as previously described.



15 The backbone in PNA compounds is two or more linked aminoethylglycine units which gives PNA an amide containing backbone. The heterocyclic base moieties are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative U.S. Patents that teach the preparation of PNA compounds include, but are not limited to, 5,539,082; 5,714,331; and 5,719,262. Further teaching of PNA compounds can be found in Nielsen et al.,  
20 Science, 1991, 254, 1497-1500.

PNA has been modified to incorporate numerous modifications since the basic PNA structure was first prepared. The basic structure is shown below:



wherein:

Bx is a heterocyclic base moiety;

T<sub>4</sub> is hydrogen, an amino protecting group, -C(O)R<sub>5</sub>, substituted or unsubstituted C<sub>1</sub>-C<sub>10</sub> alkyl, substituted or unsubstituted C<sub>2</sub>-C<sub>10</sub> alkenyl, substituted or unsubstituted C<sub>2</sub>-C<sub>10</sub> alkynyl, alkylsulfonyl, arylsulfonyl, a chemical functional group, a reporter group, a conjugate group, a D or L α-amino acid linked via the α-carboxyl group or optionally through the ω-carboxyl group when the amino acid is aspartic acid or glutamic acid or a peptide derived from D, L or mixed D and L amino acids linked through a carboxyl group, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl;

10 T<sub>5</sub> is -OH, -N(Z<sub>1</sub>)Z<sub>2</sub>, R<sub>5</sub>, D or L α-amino acid linked via the α-amino group or optionally through the ω-amino group when the amino acid is lysine or ornithine or a peptide derived from D, L or mixed D and L amino acids linked through an amino group, a chemical functional group, a reporter group or a conjugate group;

Z<sub>1</sub> is hydrogen, C<sub>1</sub>-C<sub>6</sub> alkyl, or an amino protecting group;

15 Z<sub>2</sub> is hydrogen, C<sub>1</sub>-C<sub>6</sub> alkyl, an amino protecting group, -C(=O)-(CH<sub>2</sub>)<sub>n</sub>-J-Z<sub>3</sub>, a D or L α-amino acid linked via the α-carboxyl group or optionally through the ω-carboxyl group when the amino acid is aspartic acid or glutamic acid or a peptide derived from D, L or mixed D and L amino acids linked through a carboxyl group;

Z<sub>3</sub> is hydrogen, an amino protecting group, -C<sub>1</sub>-C<sub>6</sub> alkyl, -C(=O)-CH<sub>3</sub>, benzyl, benzoyl, 20 or -(CH<sub>2</sub>)<sub>n</sub>-N(H)Z<sub>1</sub>;

each J is O, S or NH;

R<sub>5</sub> is a carbonyl protecting group; and

n is from 2 to about 50.

Another class of oligonucleotide mimetic that has been studied is based on linked 25 morpholino units (morpholino nucleic acids) having heterocyclic base moieties attached to the morpholino ring. There are a number of linking groups reported that are used to link the morpholino rings. A suitable class of linking groups were selected as being non-ionic. The non-ionic morpholino-based oligomeric compounds are less likely to have undesired interactions with cellular proteins (Dwayne A. Braasch and David R. Corey, Biochemistry, 2002, 41(14), 4503-30 4510). Morpholino-based oligomeric compounds are disclosed in U.S. Patent No. 5,034,506.

Chimeric oligomers comprising PNA and oligonucleotide sequences may be prepared by first synthesizing each of the PNA and oligonucleotide sequences separately, and then joining

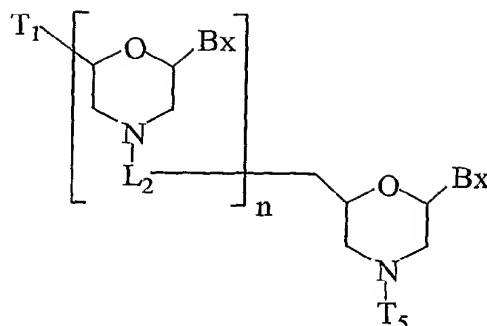
the two sequences by a suitable linking group. Alternatively, the oligonucleotide portion may be prepared on a suitable support and then the PNA portion may be synthesized in a known manner using the oligonucleotide as a primer support, optionally using a linker between the two portions. In other embodiments, the PNA portion may be prepared on a solid support by known methods, and then the oligonucleotide portion may be synthesized using the PNA portion as a linker, again optionally employing a linker between the two portions as a spacer. Suitable spacers include those known in the art to have multiple functional groups capable of reacting with the functional groups present at the termini of PNA and oligonucleotide compounds, e.g. dicarboxylic acids and dicarboxylic anhydrides such as succinic anhydride.

10 PNA-Oligonucleotide chimeras may comprise a 5'-HO-Oligonucleotide-3'-PNA motif (O-PNA hemimer motif), a PNA-5'-Oligonucleotide-3'-OH motif (PNA-O hemimer motif), a PNA-5'-Oligonucleotide-3'-PNA (PNA-O-PNA gapmer) motif, a 5'-HO-Oligonucleotide-3'-PNA-5'-Oligonucleotide-3'-OH (O-PNA-O) motif, and the like. One skilled in the art will recognize that a motif employing a PNA on a wing (end) of a hemimer or on the wings (ends) of a gapmer and a phosphate DNA oligonucleotide sequence on the other wing (end) of the hemimer, or in the gap (middle) of a gapmer, will provide the excellent nuclease resistance of PNA and the RNase H activation of a DNA oligonucleotide. For best results, at least one PNA, or at least two PNA subunits should be employed on the wing of a hemimer, or on the wings of a gapmer. Also, for superior nuclease resistance the oligonucleotide portion should have at least one phosphorothioate linker. For superior RNase H activation, there should be a minimum of about 6, or at least about 8, DNA residues in the gap of a gapmer or on the oligonucleotide wing of a hemimer. In specific embodiments of the invention, gapmers comprise 2 or 3 PNA subunits in the wings, a completely phosphorothioate DNA backbone of about 8 to about 12 DNA subunits in the gap. In other embodiments of the invention, hemimers comprise about 2 or 3 PNA subunits in one wing and a completely phosphorothioate DNA backbone of about 8 to about 12 DNA subunits on the other wing.

### *Morpholino Oligomers*

The morpholino class of oligomeric compounds have been prepared having a variety of different linking groups ( $L_2$ ) joining the monomeric subunits. The formula of the basic morpholino oligomeric compound is shown below:





wherein

$T_1$  is hydroxyl or a protected hydroxyl;

$T_5$  is hydrogen or a phosphate or phosphate derivative;

5  $L_2$  is a linking group; and

$n$  is from 2 to about 50.

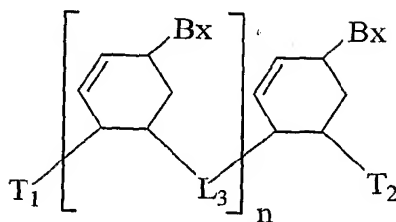
Morpholino-based oligomeric compounds are non-ionic mimics of oligonucleotides which are less likely to form undesired interactions with cellular proteins (Dwayne A. Braasch and David R. Corey, *Biochemistry*, 2002, 41(14), 4503-4510). Morpholino-based oligomeric  
10 compounds are disclosed in U.S. Patent 5,034,506.

Morpholino-Oligonucleotide chimeras may be formed as e.g. hemimers or gapmers as described in detail with regard to PNA, above, by analogous methods.

#### *CeNA Oligomers and Chimeras*

15 A further class of oligonucleotide mimetic is referred to as cyclohexenyl nucleic acids (CeNA). The furanose ring normally present in an DNA/RNA molecule is replaced with a cyclohexenyl ring. CeNA DMT protected phosphoramidite monomers have been prepared and used for oligomeric compound synthesis following classical phosphoramidite chemistry. Fully modified CeNA oligomeric compounds and oligonucleotides having specific positions modified  
20 with CeNA have been prepared and studied (see Wang et al., *J. Am. Chem. Soc.*, 2000, 122, 8595-8602). In general the incorporation of CeNA monomers into a DNA chain increases its stability of a DNA/RNA hybrid. CeNA oligoadenylates formed complexes with RNA and DNA complements with similar stability to the native complexes. The study of incorporating CeNA structures into natural nucleic acid structures was shown by NMR and circular dichroism to  
25 proceed with easy conformational adaptation. Furthermore the incorporation of CeNA into a sequence targeting RNA was stable to serum and able to activate E. Coli RNase resulting in cleavage of the target RNA strand.

The general formula of CeNA is shown below:



wherein:

each Bx is a heterocyclic base moiety;

5 T<sub>1</sub> is hydroxyl or a protected hydroxyl; and

T<sub>2</sub> is hydroxyl or a protected hydroxyl.

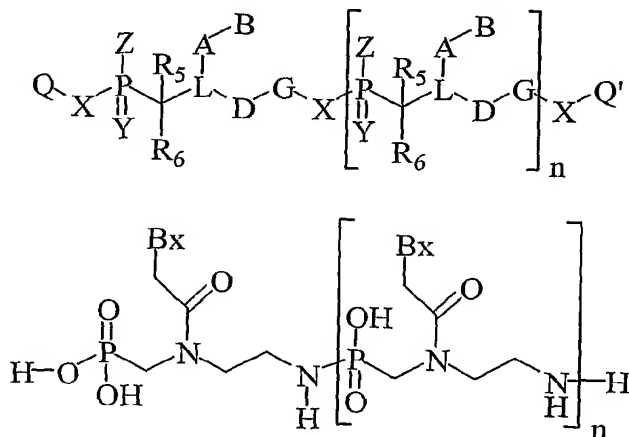
Chimeric compounds of CeNA and Oligonucleotides may be prepared as hemimers or gapmers as described above, by analogous methods.

RNAi and siRNA are related antisense modes of action, in which double stranded RNA-  
 10 like oligomers interact with mRNA to accomplish gene silencing. Preparation of double stranded oligomers progresses precisely as with single stranded oligomers, except that the two strands are prepared separately and then annealed to form the double stranded species. In some embodiments, two strands of RNA are prepared separately and the two strands are combined while the 2'-OH protecting group is still on the RNA. After the two strands are combined, the 2'-  
 15 protecting groups are removed (if necessary), and the two strands are permitted to anneal to form the double stranded species. In some cases, the two strands are prepared with 1-4 nucleoside overhangs – that is termini which do not hybridize to the opposite strand. In specific embodiments, the overhangs may be 1 or 2 nucleosides in length.

## 20 *Other Oligonucleotide Mimetics*

Another class of oligonucleotide mimetic is referred to as phosphonomonoester nucleic acids which in some embodiments have a similarity to PNA but incorporate a phosphorus group in the backbone. This class of oligonucleotide mimetic is reported to have useful physical and biological and pharmacological properties in the areas of inhibiting gene expression (antisense  
 25 oligonucleotides, ribozymes, sense oligonucleotides and triplex-forming oligonucleotides), as probes for the detection of nucleic acids and as auxiliaries for use in molecular biology. In cases where one or more of the nucleoside analogs is an LNA, the oligonucleotide mimetic may act as an LNAzyme, which may be thought of as a modified ribozyme, having the advantages of LNA, as discussed herein.

The general formula (for definitions of Markush variables see: U.S. Patent Nos. 5,874,553 and 6,127,346) is shown below along with one selection of Markush variables which give a compound having a resemblance to PNA.



5 Additional modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide and the 5' position of 5' terminal nucleotide. For example, one additional modification of the ligand conjugated oligonucleotides of the present invention involves chemically linking to the oligonucleotide one or more additional non-ligand moieties or conjugates which enhance the activity, cellular distribution or  
10 cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., Proc. Natl. Acad. Sci. USA, 1989, 86, 6553), cholic acid (Manoharan et al., Bioorg. Med. Chem. Lett., 1994, 4, 1053), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., Ann. N.Y. Acad. Sci., 1992, 660, 306; Manoharan et al., Bioorg. Med. Chem. Lett., 1993, 3, 2765), a thiocholesterol (Oberhauser et al., Nucl. Acids Res., 1992,  
15 20, 533), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., EMBO J., 1991, 10, 111; Kabanov et al., FEBS Lett., 1990, 259, 327; Svinarchuk et al., Biochimie, 1993, 75, 49), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651; Shea et al., Nucl. Acids Res., 1990, 18, 3777), a polyamine or a polyethylene glycol  
20 chain (Manoharan et al., Nucleosides & Nucleotides, 1995, 14, 969), or adamantane acetic acid (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651), a palmityl moiety (Mishra et al., Biochim. Biophys. Acta, 1995, 1264, 229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., J. Pharmacol. Exp. Ther., 1996, 277, 923).

Representative U.S. patents that teach the preparation of such oligonucleotide  
25 conjugates include, but are not limited to, U.S. Patent Nos. 4,828,979; 4,948,882; 5,218,105;

5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,580,731; 5,591,584;  
5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046;  
4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335;  
4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136;  
5 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241,  
5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810;  
5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941.

### *Oligomer Design Considerations*

10 In naturally occurring oligonucleotides, the sugar ring is  $\beta$ -D-ribosyl (RNA) or  $\beta$ -D-2'-deoxyribosyl (DNA). The hybridization behavior of DNA with RNA differs from the hybridization of RNA to RNA. This difference gives rise to different *in vitro* and *in vivo* effects. For example, DNA-RNA hybrids effectively bind to RNase H, which results in scission of RNA. In contrast, RNA-RNA hybrids may be unwound by helicase, whereby the antisense  
15 strand is permitted to form a hybrid with mRNA. The exogenous RNA-mRNA hybrid interacts with one or more members of the RISC complex, which effects mRNA scission.

Synthetic sugars and sugar analogs are designed to adopt certain spatial conformations that resemble DNA, RNA or some structure intermediate between these conformations. Again, the sugar or sugar analog functions as a sort of platform to hold the base in the correct  
20 orientation to interact with bases on the opposite strand. The sugar or sugar analog (collectively skeletal members) also provides binding sites for the linking groups, which join the monomeric units together to form the oligomer. The conformation of the sugar or sugar analog greatly influences the spatial orientations of the bases and linking groups, and also greatly influences the shape of the antisense-sense hybrid in solution. This conformational influence can have an  
25 important impact on the efficacy of the antisense compound in modulation of gene expression.

In the broadest sense, the term "oligonucleotide" refers to an oligomer having a plurality of skeletal members, e.g. sugar units (ribosyl, deoxyribosyl, arabinosyl, modified sugar unit, etc.) linked by phosphate diester linkers (i.e. phosphoryl or thiophosphoryl diester), and having bases for establishing binding to complementary oligomer strands. In some embodiments  
30 of the invention, an oligonucleotide may contain both phosphoryl diester and phosphorothioate linkers. In other embodiments, the linkers are all phosphorothioate linkers. While phosphoryl linkers are the naturally occurring type of linkers in oligonucleotides, thiophosphate linkers are known to confer nuclease stability to oligonucleotides cells. Hence, it is often suitable to prepare

oligonucleotides with at least a portion of the phosphate diester moieties replaced by phosphorothioate diester moieties.

As described herein, oligonucleotides can be prepared as chimeras with other oligomeric moieties. In the context of this invention, the term "oligomeric compound" refers to a polymeric structure capable of hybridizing a region of a nucleic acid molecule, and an "oligomeric moiety" a portion of such an oligomeric compound. Oligomeric compounds include oligonucleotides, oligonucleosides, oligonucleotide analogs, modified oligonucleotides and oligonucleotide mimetics. Oligomeric compounds can be linear or circular, and may include branching. They can be single stranded or double stranded, and when double stranded, may include overhangs. In general, an oligomeric compound comprises a backbone of linked monomeric subunits where each linked monomeric subunit is directly or indirectly attached to a heterocyclic base moiety. The linkages joining the monomeric subunits, the monomeric subunits and the heterocyclic base moieties can be variable in structure giving rise to a plurality of motifs for the resulting oligomeric compounds including hemimers, gapmers and chimeras. As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base moiety. The two most common classes of such heterocyclic bases are purines and pyrimidines. In the context of this invention, the term "oligonucleoside" refers to nucleosides that are joined by internucleoside linkages that do not have phosphorus atoms. Internucleoside linkages of this type include short chain alkyl, cycloalkyl, mixed heteroatom alkyl, mixed heteroatom cycloalkyl, one or more short chain heteroatomic and one or more short chain heterocyclic. These internucleoside linkages include, but are not limited to, siloxane, sulfide, sulfoxide, sulfone, acetyl, formacetyl, thioformacetyl, methylene formacetyl, thioformacetyl, alkeneyl, sulfamate; methyleneimino, methylenehydrazino, sulfonate, sulfonamide, amide and others having mixed N, O, S and CH<sub>2</sub> component parts.

## 25 *Uses for Oligomers*

Oligomers, and especially oligonucleotides and chimeras according to the present invention, have been used in a variety of applications, including in assays, sequence arrays, primers and probes for nucleic acid amplification (e.g. PCR), as antisense molecules for gene target validation and therapeutic applications, etc. The person skilled in the art will recognize that the methods according to the present invention may be adapted to prepare oligomers for such applications. Accordingly, only select uses of oligomers according to the present invention will be described herein.

Exemplary antisense compounds include DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 5'-terminus of one of the illustrative antisense compounds (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately upstream of the 5'-terminus of the antisense compound which is specifically hybridizable to the target nucleic acid and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). Similarly suitable antisense compounds are represented by DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 3'-terminus of one of the illustrative antisense compounds (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately downstream of the 3'-terminus of the antisense compound which is specifically hybridizable to the target nucleic acid and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). One having skill in the art, once armed with the empirically-derived suitable antisense compounds illustrated herein will be able, without undue experimentation, to identify further suitable antisense compounds.

Antisense and other compounds of the invention, which hybridize to the target and inhibit expression of the target, are identified through experimentation, and representative sequences of these compounds are herein identified as embodiments of the invention. While specific sequences of the antisense compounds are set forth herein, one of skill in the art will recognize that these serve to illustrate and describe particular embodiments within the scope of the present invention. Additional antisense compounds may be identified by one having ordinary skill.

Specific examples of suitable antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Double stranded oligomers according to the present invention may be employed via a mode of antisense activity known as RNAi or siRNA. In RNAi, long, double stranded RNA or RNA mimetic oligomer is cleaved by the dicer enzyme to form double stranded RNA of about 21 nt (21 nucleotides) in length. In siRNA, short, hybridized sense-antisense pairs of RNA or RNA mimetic oligomer are directly introduced into a cell. In either case, the short, double-

stranded RNA or RNA mimetic oligomer interacts with an intracellular agent, such as a helicase, capable of unraveling the double-stranded oligomer to produce a free antisense oligomer. This antisense oligomer then interacts with the RISC complex and its target nucleotide (generally mRNA) to cleave the target nucleotide. The cleaved nucleotide is thus rendered incapable of  
5 being translated, and a form of gene silencing is effected thereby. It is considered that oligomers according to the present invention may be prepared as siRNA duplexes, with or without a 1-4 nt overhang on each end, for use in siRNA. It is also considered possible to prepare stabilized, single-stranded RNA mimetic that will activate the RISC complex for target-selective gene silencing. Accordingly, oligomers comprising a double-stranded or single-stranded motif  
10 capable of activating the RISC complex for gene silencing are considered to be antisense agents within the scope of the present invention.

Expression patterns within cells or tissues treated with one or more antisense compounds are compared to control cells or tissues not treated with antisense compounds and the patterns produced are analyzed for differential levels of gene expression as they pertain, for  
15 example, to disease association, signaling pathway, cellular localization, expression level, size, structure or function of the genes examined. These analyses can be performed on stimulated or unstimulated cells and in the presence or absence of other compounds which affect expression patterns.

Examples of methods of gene expression analysis known in the art include DNA arrays  
20 or microarrays (Brazma and Vilo, FEBS Lett., 2000, 480, 17-24; Celis, et al., FEBS Lett., 2000, 480, 2-16), SAGE (serial analysis of gene expression)(Madden, et al., Drug Discov. Today, 2000, 5, 415-425), READS (restriction enzyme amplification of digested cDNAs) (Prashar and Weissman, Methods Enzymol., 1999, 303, 258-72), TOGA (total gene expression analysis) (Sutcliffe, et al., Proc. Natl. Acad. Sci. U. S. A., 2000, 97, 1976-81), protein arrays and  
25 proteomics (Celis, et al., FEBS Lett., 2000, 480, 2-16; Jungblut, et al., Electrophoresis, 1999, 20, 2100-10), expressed sequence tag (EST) sequencing (Celis, et al., FEBS Lett., 2000, 480, 2-16; Larsson, et al., J. Biotechnol., 2000, 80, 143-57), subtractive RNA fingerprinting (SuRF) (Fuchs, et al., Anal. Biochem., 2000, 286, 91-98; Larson, et al., Cytometry, 2000, 41, 203-208), subtractive cloning, differential display (DD) (Jurecic and Belmont, Curr. Opin. Microbiol.,  
30 2000, 3, 316-21), comparative genomic hybridization (Carulli, et al., J. Cell Biochem. Suppl., 1998, 31, 286-96), FISH (fluorescent in situ hybridization) techniques (Going and Gusterson, Eur. J. Cancer, 1999, 35, 1895-904) and mass spectrometry methods (reviewed in To, Comb. Chem. High Throughput Screen, 2000, 3, 235-41).

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to humans and numerous clinical trials  
5 are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

It is suitable to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process.  
10 The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding a particular protein. The targeting process also includes determination of a site or sites  
15 within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, one intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the  
20 corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon." A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in  
25 each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are  
30 used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding a particular protein, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding



DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a suitable target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be suitable target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also suitable targets. mRNA transcripts produced via the process of splicing of two (or more) mRNAs from different gene sources are known as "fusion transcripts." It has also been found that introns can be effective, and therefore suitable, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

It is also known in the art that alternative RNA transcripts can be produced from the same genomic region of DNA. These alternative transcripts are generally known as "variants."

More specifically, "pre-mRNA variants" are transcripts produced from the same genomic DNA that differ from other transcripts produced from the same genomic DNA in either their start or stop position and contain both intronic and extronic regions.

Upon excision of one or more exon or intron regions or portions thereof during splicing, 5 pre-mRNA variants produce smaller "mRNA variants." Consequently, mRNA variants are processed pre-mRNA variants and each unique pre-mRNA variant must always produce a unique mRNA variant as a result of splicing. These mRNA variants are also known as "alternative splice variants." If no splicing of the pre-mRNA variant occurs then the pre-mRNA variant is identical to the mRNA variant.

10 It is also known in the art that variants can be produced through the use of alternative signals to start or stop transcription and that pre-mRNAs and mRNAs can possess more than one start codon or stop codon. Variants that originate from a pre-mRNA or mRNA that use alternative start codons are known as "alternative start variants" of that pre-mRNA or mRNA. Those transcripts that use an alternative stop codon are known as "alternative stop variants" of 15 that pre-mRNA or mRNA. One specific type of alternative stop variant is the "polyA variant" in which the multiple transcripts produced result from the alternative selection of one of the "polyA stop signals" by the transcription machinery, thereby producing transcripts that terminate at unique polyA sites.

Once one or more target sites have been identified, oligonucleotides are chosen which 20 are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary 25 nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The 30 oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such

that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable.

An antisense compound is specifically hybridizable when binding of the compound to  
5 the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of activity, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment, and in the case of in vitro assays, under conditions in which the assays are  
10 performed. It is suitable that the antisense compounds of the present invention comprise at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, or at least 100% sequence complementarity with the target nucleic acid sequence to which they are targeted. Percent complementarity of an antisense compound with a target nucleic acid can be determined routinely using basic local alignment search tools (BLAST programs) (Altschul et al., J. Mol.  
15 Biol., 1990, 215, 403-410; Zhang and Madden, Genome Res., 1997, 7, 649-656).

Antisense and other compounds of the invention, which hybridize to the target and inhibit expression of the target, are identified through experimentation, and representative sequences of these compounds are hereinbelow identified as suitable embodiments of the invention. The sites to which these suitable antisense compounds are specifically hybridizable  
20 are hereinbelow referred to as "suitable target regions" and are therefore suitable sites for targeting. As used herein the term "suitable target region" is defined as at least an 8-nucleobase portion of a target region to which an active antisense compound is targeted. While not wishing to be bound by theory, it is presently believed that these target regions represent regions of the target nucleic acid which are accessible for hybridization.

25 While the specific sequences of particular suitable target regions are set forth below, one of skill in the art will recognize that these serve to illustrate and describe particular embodiments within the scope of the present invention. Additional target regions may be identified by one having ordinary skill.

Target regions 8-80 nucleobases in length comprising a stretch of at least eight (8)  
30 consecutive nucleobases selected from within the illustrative target regions are considered to be suitable suitable target regions as well.

Exemplary target regions include DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 5'-terminus of one of the illustrative target regions (the

remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately upstream of the 5'-terminus of the target region and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). Similarly suitable target regions are represented by DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 3'-  
5 terminus of one of the illustrative target regions (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately downstream of the 3'-terminus of the target region and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). One having skill in the art, once armed with the empirically-derived suitable target regions illustrated herein will be able, without undue experimentation, to identify further target regions.  
10 In addition, one having ordinary skill in the art will also be able to identify additional compounds, including oligonucleotide probes and primers, that specifically hybridize to these target regions using techniques available to the ordinary practitioner in the art.

The ability of oligonucleotides to bind to their complementary target strands is compared by determining the melting temperature ( $T_m$ ) of the hybridization complex of the  
15 oligonucleotide and its complementary strand. The melting temperature ( $T_m$ ), a characteristic physical property of double helices, denotes the temperature (in degrees centigrade) at which 50% helical (hybridized) versus coil (unhybridized) forms are present.  $T_m$  is measured by using the UV spectrum to determine the formation and breakdown (melting) of the hybridization complex. Base stacking, which occurs during hybridization, is accompanied by a reduction in  
20 UV absorption (hypochromicity). Consequently, a reduction in UV absorption indicates a higher  $T_m$ . The higher the  $T_m$ , the greater the strength of the bonds between the strands. The structure-stability relationships of a large number of nucleic acid modifications have been reviewed (Freier and Altmann, Nucl. Acids Research, 1997, 25, 4429-443).

In the case of SARS, it is considered that the directing of antisense agents to one or  
25 more of the 11 open reading frames will be successful in attenuating the viability of the virus, e.g. by interfering with replication of the viral genome, translation of one or more structural proteins, by interfering with the virus' ability to assemble or exit the host cell, etc. The genome of SARS-CoV is a 29,727-nucleotide polyadenylated RNA, and 41% of the residues are G or C. The genomic organization is typical of coronaviruses, with the characteristic gene order (5'-  
30 replicase [rep], spike [S], envelope [E], membrane [M], nucleocapsid [N]-3' and short untranslated regions at both termini. The SARS-CoV rep gene, which comprises about two-thirds of the genome, is predicted to encode two polyproteins that undergo co-translational proteolytic processing. There are four open reading frames (ORFs) downstream of rep that are predicted to

encode the structural proteins, S, E, M and N, which are common to all known coronaviruses. It is considered that directing the antisense agent to S, E, M, N or rep itself will succeed in interfering with the ability of the virus to express one or more proteins vital to the viral replication cycle. In particular, it is considered that an antisense agent to rep will likely be  
5 successful in interfering with the viability of SARS-CoV, as attenuation of rep expression should result in failure of the virus to replicate its viral genome. It is also considered that antisense agents to one or more of S, E, M and N may interfere with viral propagation, as such structural genes are likely to be critical to proper assembly of the intact virion before it exits the host cell.

In order that the invention disclosed herein may be more efficiently understood,  
10 examples are provided below. It should be understood that these examples are for illustrative purposes only and are not to be construed as limiting the invention in any manner. Throughout these examples, molecular cloning reactions, and other standard recombinant DNA techniques, were carried out according to methods described in Maniatis et al., Molecular Cloning - A Laboratory Manual, 2nd ed., Cold Spring Harbor Press (1989), using commercially available  
15 reagents, except where otherwise noted.

## EXAMPLES

### Example 1: Preparation of Antisense Oligonucleotide

A fully phosphorothioate anti-SARS antisense oligonucleotide is prepared according to  
20 known methods as modified herein. Generally, the synthesis may be performed on a suitable automated synthesizer, e.g. the Pharmacia OligoPilot II Synthesizer on a suitable scale, such as a 120 micromole scale, using p-methoxy phosphoramidites and a suitable solid support, e.g. Pharmacia's Primer HL30™ solid support. Detritylation may be performed using a weak acid solution, e.g. a 3% dichloroacetic acid in toluene (volume/volume) or a 10% dichloroacetic acid  
25 solution in toluene. The support may then be washed with acetonitrile, and then a 0.2 M solution of phenylacetyl disulfide in 3-picoline:acetonitrile (1:1) added and allowed to react at room temperature for 2 minutes to form a support-bound phosphorothioate triester. At the end of synthesis, the support-bound oligonucleotide may be treated first with a solution of 2-methyl-5-tert-butylthiophenol:triethylamine:acetonitrile (1:1:3) for 2 hours to remove the methyl  
30 phosphorus protecting groups, and then with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature and then incubated at 55°C for 16 hours to remove the oligonucleotide from the solid support. The oligonucleotide may be purified by, e.g. RP HPLC,

removal of the ultimate trityl group, followed by desalting, lyophilization and optionally reconstitution.

#### **Example 2: Synthesis of Gapmer**

5       The same method employed in Example 1 is used to make a 5-10-5 gapmer, in which the wings are 2'-O-methoxyethyl nucleosides (MOE nucleoside) and the gap is fully 2'-deoxy nucleoside, except that the phosphoramidites primer support comprises a MOE nucleoside bound to the support via a reversible linker, and the phosphoramidites used on the wings are 2'-O-methoxyethyl modified phosphoramidites.

10

#### **Example 3: Synthesis of siRNA Oligomer**

      The same method employed in Example 1 is used to make a double stranded RNA, except that the two strands are made in side-by-side tandem synthesis, the phosphoramidites primer support comprises a 2'-O-protected ribose bound to the support via a reversible linker, the  
15 phosphoramidites used on the wings are 2'-O-protected ribosyl phosphoramidites, and after cleavage from the support, the two strands are combined, the 2'-protecting groups are removed, and the strands are permitted to hybridize to form the double stranded species.

#### **Example 4: Preparation of Antisense Agents for Pulmonary Administration**

20       An oligomer according to one of examples 1-3 may be prepared as a solid or liquid preparation for aerosolization, nebulization, metered dosing, intranasal instillation, etc. according to methods known in the art. See, for example, U.S. Patent Nos. 6,518,239, 6,303,582, 6,503,480, 6,433,040, 6,509,006, 6,372,258, 6,349,719, 6,167,880, 6,098,620, 5,957,124, 5,906,202, 5,819,726, 5,755,218, 5,522,385, 6,447,753, 6,387,390, 5,985,320, 5,985,309,  
25 5,855,913, 6,550,472, 6,543,443, 6,540,154, 6,467,476, 6,427,682, 6,543,448, 6,546,929 and 5,049,388. The solid or liquid formulation is then administered to a patient in need thereof, at a dosage range suitable for treatment of SARS. As a general matter, the minimum dose will be used to elicit the desired effect, e.g. attenuation of viral load in the affected tissue(s), amelioration of syndrome symptoms, etc. Daily doses in the range of about 0.01 µg to about  
30 1000 mg per day are within the scope of the invention. In some cases, the ranges are in the range of about 0.01 µg to about 10 mg per day, and in particular about 0.01 µg to about 5 mg per day. The dosage may vary by weight of the patient, severity of the syndrome, viral load in the lung,

lung breathing capacity (i.e. volume at full pulmonary expansion), presence or absence of other disease states (e.g. emphysema, pneumonia, cancer, etc.), age and sex of the patient, etc.

#### **Example 5: Preparation of Nucleoside or Nucleoside Mimetic for Pulmonary Administration**

A nucleoside or nucleoside mimetic as set forth in Appendix 6 is prepared by a known method. The nucleoside or nucleoside mimetic is then prepared in a form suitable for pulmonary or nasal administration, as set forth herein. See for example U.S. Patent Nos. 6,518,239, 6,303,582, 6,503,480, 6,433,040, 6,509,006, 6,372,258, 6,349,719, 6,167,880, 6,098,620, 10 5,957,124, 5,906,202, 5,819,726, 5,755,218, 5,522,385, 6,447,753, 6,387,390, 5,985,320, 5,985,309, 5,855,913, 6,550,472, 6,543,443, 6,540,154, 6,467,476, 6,427,682, 6,543,448, 6,546,929 and 5,049,388. The solid or liquid formulation is then administered to a patient in need thereof, at a dosage range suitable for treatment of SARS. As a general matter, the minimum dose will be used to elicit the desired effect, e.g. attenuation of viral load in the 15 affected tissue(s), amelioration of syndrome symptoms, etc. Daily doses in the range of about 0.01  $\mu$ g to about 1000 mg per day are within the scope of the invention. In some cases, the ranges are in the range of about 0.01  $\mu$ g to about 10 mg per day, and in particular about 0.01  $\mu$ g to about 5 mg per day. The dosage may vary by weight of the patient, severity of the syndrome, viral load in the lung, lung breathing capacity (i.e. volume at full pulmonary expansion), 20 presence or absence of other disease states (e.g. emphysema, pneumonia, cancer, etc.), age and sex of the patient, etc.

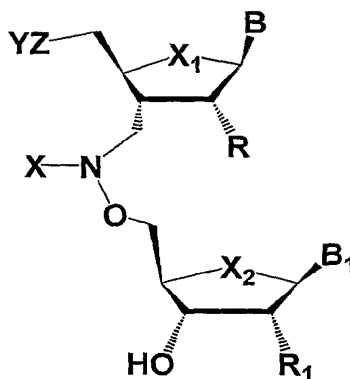
#### **Example 6: Preparation of Antiviral Compound for Nasal Administration.**

An antisense agent per one of examples 1-3, or an antiviral nucleoside, nucleoside 25 mimetic or dimer according to one of examples 4 or 5 may be prepared according to one of the methods known in the art. See, e.g. U.S. Patent Nos. 6,551,578, 6,554,497, 6,485,707, 6,468,507, 6,464,959, 6,294,153, 6,214,805, 6,087,343, 5,985,320, and 5,744,166. Administration is via dropper, spray, and the like. Dosing is as per examples 1-5 above.

#### **30 Example 7: Representative Antiviral Dimers**

The following represents suitable antiviral dimers for employment in the compositions and methods described herein.

MMI



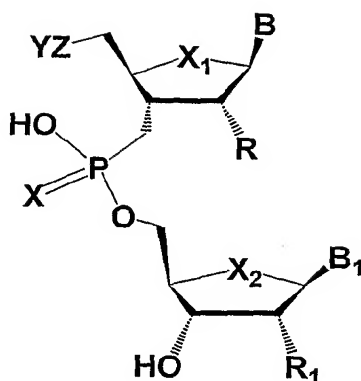
wherein:

- 5 each of B and B<sub>1</sub> are, independently, adenine (A), 7-deaza A, guanine (G), 7-deaza G, Cytosine (C), 5MeC, uracil (U), 5-MeU, 2-Amino A, 2,6-diamino A, or a substituted or unsubstituted purine, pyrimidine, or pyridine;
- R and R<sub>1</sub> are, independently or in combination, H, OH, OCH<sub>3</sub>, OCH<sub>2</sub>OCH<sub>3</sub>, F, Cl, Br, I, NH<sub>2</sub>, alkylaminooxyalkyl, or haloalkyl;
- 10 Y is a phosphate, protected phosphate, phosphate mimic, or boranophosphate;  
Z is O, S, or CH<sub>2</sub>;
- X<sub>1</sub> and X<sub>2</sub> are, independently, O, S, CH<sub>2</sub>, CHF, CF<sub>2</sub>, CHOH, NH, or NR<sub>2</sub>; and  
R<sub>2</sub> is C<sub>1</sub>-C<sub>6</sub> alkyl or a carbonyl.
- X is H, OH, NH<sub>2</sub>, lower alkyl, substituted lower alkyl, alkoxy, lower alkenyl, aralkyl,  
15 alkylamino, aralkylamino, substituted alkylamino, heterocycloalkyl, heterocycloalkylamino, aminoalkylamino, polyalkylamino, an RNA cleaving group, a group for improving the pharmacokinetic properties of an oligonucleotide or a group for improving the pharmacodynamic properties of an oligonucleotide. See, U.S. Patent No. 5,378,825.

20

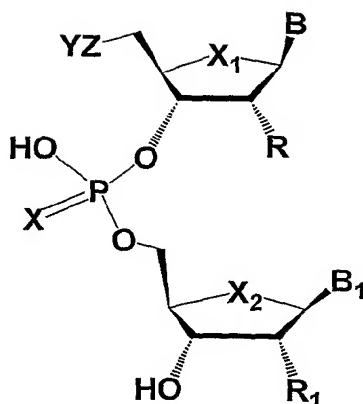
25



*3'-C-Phosphate Dimers*

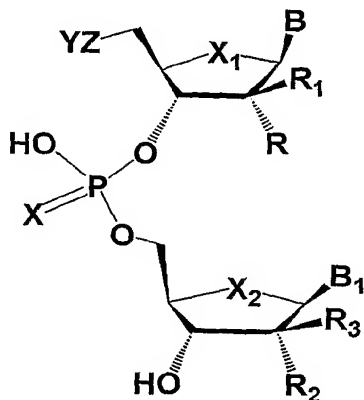
wherein:

- 5 each of B and B<sub>1</sub> are, independently, adenine (A), 7-deaza A, guanine (G), 7-deaza G, Cytosine (C), 5MeC, uracil (U), 5-MeU, 2-Amino A, 2,6-diamino A, or a substituted or unsubstituted purine, pyrimidine, or pyridine;
- X is O or S;
- R and R<sub>1</sub> are, independently or in combination, H, OH, OCH<sub>3</sub>, OCH<sub>2</sub>OCH<sub>3</sub>, F, Cl, Br, I,
- 10 NH<sub>2</sub>, alkylaminooxyalkyl, or haloalkyl;
- Y is a phosphate, protected phosphate, phosphate mimic, or boranophosphate;
- Z is O, S, or CH<sub>2</sub>;
- X<sub>1</sub> and X<sub>2</sub> are, independently, O, S, CH<sub>2</sub>, CHF, CF<sub>2</sub>, CHOH, NH, or NR<sub>2</sub>; and
- R<sub>2</sub> is C<sub>1</sub>-C<sub>6</sub> alkyl or a carbonyl.
- 15 Methods of making the MMI and 3'-C-phosphate dimers are set forth in, *inter alia*, U.S. Patent No. 5,378,825. In particular, Examples 13-20 of U.S. Patent No. 5,378,825 demonstrate methods of making MMI and 3'-C-phosphate dimer compounds. Dimer compounds may also be manufactured by solution phase reaction, analogous to the solid phase synthesis set forth in the
- 20 other 3'-protecting group for the linker to the solid support at the 3'-OH of the 3'-side nucleoside.

*Phosphodiesters*

wherein:

- 5 each of B and B<sub>1</sub> are, independently, adenine (A), 7-deaza A, guanine (G), 7-deaza G, Cytosine (C), 5MeC, uracil (U), 5-MeU, 2-Amino A, 2,6-diamino A, or a substituted or unsubstituted purine, pyrimidine, or pyridine;
- X is O or S;
- R and R<sub>1</sub> are, independently or in combination, H, OH, OCH<sub>3</sub>, OCH<sub>2</sub>OCH<sub>3</sub>, F, Cl, Br, I,
- 10 NH<sub>2</sub>, alkylaminooxyalkyl, or haloalkyl;
- Y is a phosphate, protected phosphate, phosphate mimic, or boranophosphate;
- Z is O, S, or CH<sub>2</sub>;
- X<sub>1</sub> and X<sub>2</sub> are, independently, O, S, CH<sub>2</sub>, CHF, CF<sub>2</sub>, CHOH, NH, or NR<sub>2</sub>; **and**
- R<sub>2</sub> is C<sub>1</sub>-C<sub>6</sub> alkyl or a carbonyl.
- 15 Phosphodiester dimers may be manufactured by synthetic methods know in the art for producing phosphodiesters, such as the methods set forth in U.S. Patent No. 6,211,350.
- Other compounds have the structure:



20 wherein:

each of B and B<sub>1</sub> are, independently, adenine (A), 7-deaza A, guanine (G), 7-deaza G, Cytosine (C), 5MeC, uracil (U), 5-MeU, 2-Amino A, 2,6-diamino A, or a substituted or unsubstituted purine, pyrimidine, or pyridine;

X is O or S;

5 R, R<sub>1</sub>, R<sub>2</sub>, and R<sub>3</sub> are, independently or in combination, H, OH, OCH<sub>3</sub>, OCH<sub>2</sub>OCH<sub>3</sub>, F, Cl, Br, I, NH<sub>2</sub>, alkylaminooxyalkyl, or haloalkyl;

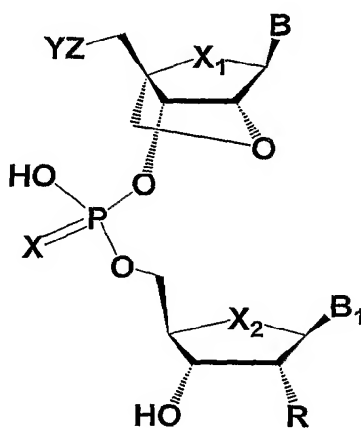
Y is a phosphate, protected phosphate, phosphate mimic, or boranophosphate;

Z is O, S, or CH<sub>2</sub>;

X<sub>1</sub> and X<sub>2</sub> are, independently, O, S, CH<sub>2</sub>, CHF, CF<sub>2</sub>, CHOH, NH, or NR<sub>2</sub>.

10

### *LNA-NA Dimers*



wherein:

each of B and B<sub>1</sub> are, independently, adenine (A), 7-deaza A, guanine (G), 7-deaza G, Cytosine (C), 5MeC, uracil (U), 5-MeU, 2-Amino A, 2,6-diamino A, or a substituted or unsubstituted purine, pyrimidine, or pyridine;

X is O or S;

R is H, OH, OCH<sub>3</sub>, OCH<sub>2</sub>OCH<sub>3</sub>, F, Cl, Br, I, NH<sub>2</sub>, alkylaminooxyalkyl, or haloalkyl;

Y is a phosphate, protected phosphate, phosphate mimic, or boranophosphate;

20 Z is O, S, or CH<sub>2</sub>;

X<sub>1</sub> and X<sub>2</sub> are, independently, O, S, CH<sub>2</sub>, CHF, CF<sub>2</sub>, CHOH, NH, or NR<sub>2</sub>; and

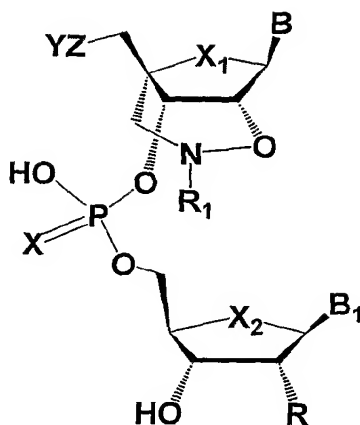
R<sub>2</sub> is C<sub>1</sub>-C<sub>6</sub> alkyl or a carbonyl.

LNA-NA dimers may be synthesized by methods known in the art. In particular, the NA monomer may be produced by known methods and the LNA monomer may be produced by known methods, such as those set forth in U.S. Patent Nos. 6,525,191 and 6,268,490. The dimers

25

may then be linked by methods known in the art, such as those taught by U.S. Patent No. 6,211,350.

*Oxazolidine LNAs (OLNA)*



wherein:

each of B and B<sub>1</sub> are, independently, adenine (A), 7-deaza A, guanine (G), 7-deaza G, Cytosine (C), 5MeC, uracil (U), 5-MeU, 2-Amino A, 2,6-diamino A, or a substituted or unsubstituted purine, pyrimidine, or pyridine;

X is O or S;

R<sub>1</sub> is H, alkyl, or aryl;

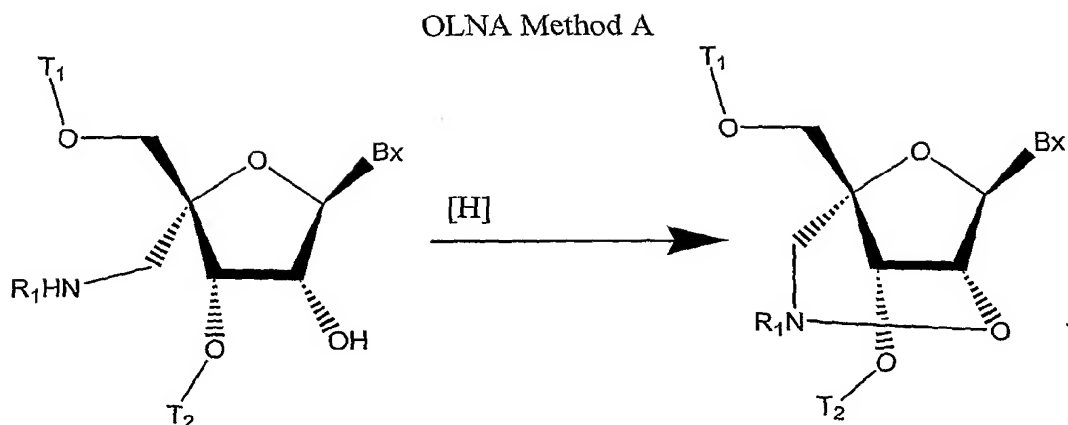
Y is a phosphate, protected phosphate, phosphate mimic, or boranophosphate;

Z is O, S, or CH<sub>2</sub>;

X<sub>1</sub> and X<sub>2</sub> are, independently, O, S, CH<sub>2</sub>, CHF, CF<sub>2</sub>, CHOH, NH, or NR<sub>2</sub>; and

R<sub>2</sub> is C<sub>1</sub>-C<sub>6</sub> alkyl or a carbonyl.

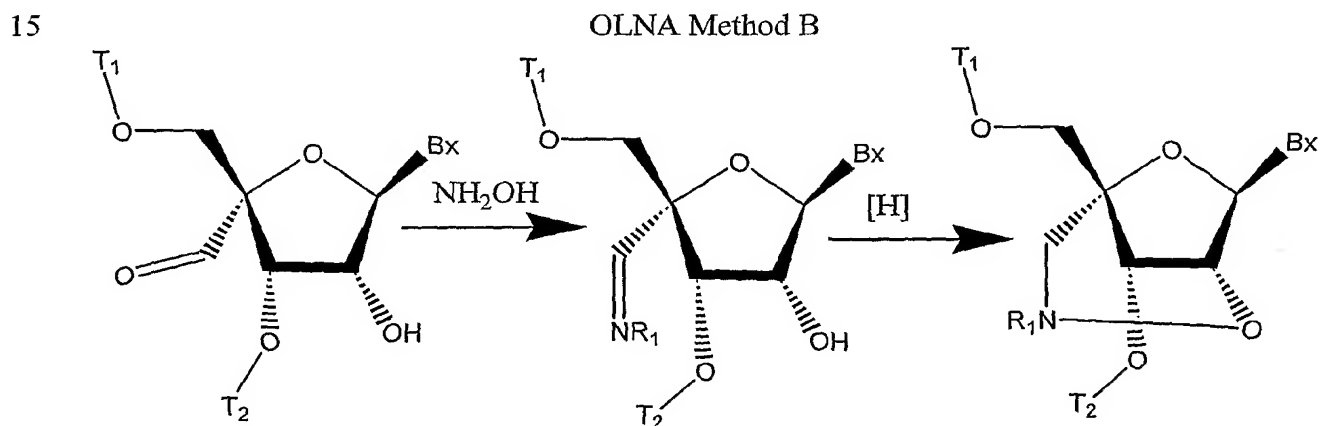
The oxazolidino nucleosides can be produced by an art recognized method, such as the reductive ring closure of a 4'-aminomethylnucleoside (OLNA Method A), or by oximation of a 4'-formyl nucleoside to the 4'-oximylmethyl, followed by reductive ring closure (OLNA Method B).



wherein:

- 5             $T_1$  and  $T_2$  are, independently, H or a suitable protecting group;  
               $R_1$  is defined above; and  
              Bx has the meaning of B.

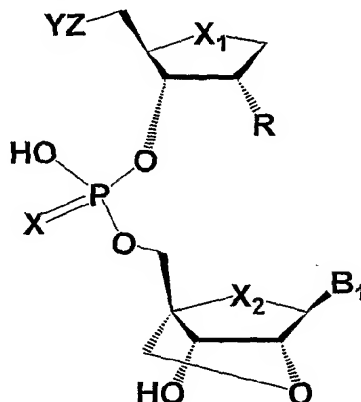
The starting materials for this reaction may be made by the method set forth in U.S. Patent No. 5,763,596, especially at scheme 3, columns 9 and 10. The reduction may be carried  
 10 out in a suitable reaction solvent under suitable conditions and in the presence of a suitable reducing agent, for example alkyl aluminum hydride, as disclosed in U.S. Patent No. 4,252,964, especially at columns 7 and 8, the discussion of the manufacture of compounds XXII and XXIII. Other reducing reagents include  $LiAlH_4$  and other metal hydrides capable of effecting the ring closure of NHR and OH to form NR-O.



The starting 4'-formyl nucleosides may be produced by the methods described in U.S. Patent No. 5,192,749. Other methods for making 4'-substituted nucleosides and oligonucleotides containing them are set forth in U.S. Patent No. 5,446,137.

Protecting groups  $T_1$  and  $T_2$  are known in the art. For example, U.S. Patent No. 5,446,137 sets forth protecting groups such as the 5'-trityl group and the 3'-tertbutyldimethylsilyl group. Other protecting groups are set forth in U.S. Patent No. 6,211,350.

Other compounds have the structure:



wherein:

each of B and  $B_1$  are, independently, adenine (A), 7-deaza A, guanine (G), 7-deaza G, Cytosine (C), 5MeC, uracil (U), 5-MeU, 2-Amino A, 2,6-diamino A, or a substituted or unsubstituted purine, pyrimidine, or pyridine;

X is O or S;

R is H, OH,  $OCH_3$ ,  $OCH_2OCH_3$ , F, Cl, Br, I,  $NH_2$ , alkylaminooxyalkyl, or haloalkyl;

Y is a phosphate, protected phosphate, phosphate mimic, or boranophosphate;

Z is O, S, or  $CH_2$ ;

$X_1$  and  $X_2$  are, independently, O, S,  $CH_2$ , CHF,  $CF_2$ , CHOH, NH, or  $NR_2$ ; and

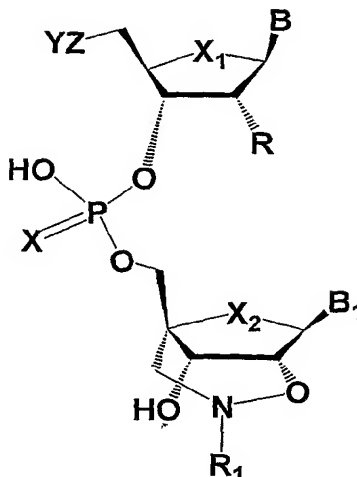
$R_2$  is  $C_1$ - $C_6$  alkyl or a carbonyl.

NA-LNA dimers may be synthesized by methods known in the art. In particular, the NA monomer may be produced by known methods and the LNA monomer may be produced by known methods, such as those set forth in U.S. Patent Nos. 6,525,191 and 6,268,490. The dimers may then be linked by methods known in the art, such as those taught in U.S. Patent No. 6,211,350.

Other references that provide useful information for making compounds according to the present invention include the following: Bhat, Balkrishen; Swayze, Eric E.; Wheeler, Patrick; Dimock, Stuart; Perbost, Michel; Sanghvi, Yogesh S. Synthesis of Novel Nucleic Acid Mimics via the Stereoselective Intermolecular Radical Coupling of 3'-Iodo Nucleosides and Formaldoximes, *J. Organic Chem.*, 1996, 61(23), 8186-8199; and Swayze, Eric E.; Sanghvi, Yogesh S. The synthesis of  $N',N'$ -O-trisubstituted hydroxylamines via a mild reductive

alkylation procedure. An improved synthesis of the MMI backbone, Synlett, 1997, (7), 859-861.

#### OLNA-NA Dimer



5 wherein:

each of B and B<sub>1</sub> are, independently, adenine (A), 7-deaza A, guanine (G), 7-deaza G, Cytosine (C), 5MeC, uracil (U), 5-MeU, 2-Amino A, 2,6-diamino A, or a substituted or unsubstituted purine, pyrimidine, or pyridine;

10 X is O or S;

R is H, OH, OCH<sub>3</sub>, OCH<sub>2</sub>OCH<sub>3</sub>, F, Cl, Br, I, NH<sub>2</sub>, alkylaminooxyalkyl, or haloalkyl;

R is H, alkyl, or aryl;

Y is a phosphate, protected phosphate, phosphate mimic, or boranophosphate;

Z is O, S, or CH<sub>2</sub>;

15 X<sub>1</sub> and X<sub>2</sub> are, independently, O, S, CH<sub>2</sub>, CHF, CF<sub>2</sub>, CHOH, NH, or NR<sub>2</sub> and

R<sub>2</sub> is C<sub>1</sub>-C<sub>6</sub> alkyl or a carbonyl.

These may be made by methods analogous to those set forth above for the LNA-NA dimers. The OLNA monomer may be made by methods as described in OLNA Methods A and B.

20

#### Example 8: Compositions and Methods for the Pulmonary Delivery of Nucleic Acids

The present invention provides compositions for the pulmonary administration of oligonucleotides that can contain carrier compounds, penetration enhancing agents, and transfection agents. However, the present invention also provides compositions and methods for  
25 the pulmonary administration of oligonucleotides that are substantially free of As used herein,

the term "substantially free of carriers or penetration enhancing agents" means that a de minimis amount (i.e., an amount less than that recognized to be effective) of carriers or penetration enhancing agents can be present in the composition. In particular, these modalities of the invention are drawn to compositions that comprise less than 10 mole percent, less than 1 mole percent, or less than 0.1 mole percent of such carriers or penetration enhancing agents.

In some embodiments, the present invention provides pharmaceutical compositions for pulmonary administration of large molecule therapeutics such as oligonucleotides comprising the oligonucleotide and at least one substance which facilitates the transport of a drug across the mucous membrane(s) of the lung (so called "mucosal penetration enhancers," also known as "absorption enhancers" or simply as "penetration enhancers"). See Muranishi, *Crit. Rev. Ther. Drug Carrier Systems*, 1990, 7:1 and Lee *et al.*, *Crit. Rev. Ther. Drug Carrier Systems*, 1991, 8:91.

The present invention provides compositions and methods for pulmonary delivery of one or more nucleic acids to an animal. For purposes of the invention, the term "animal" is meant to encompass humans as well as other mammals, as well as reptiles, fish, amphibians, and birds. The term "pulmonary delivery" refers to the administration, directly or otherwise, to a portion of the lung of an animal. The term "lung" has its accustomed meaning as the chief organ of respiration (i.e. gas exchange) in an animal. As used herein, the term "pulmonary delivery" subsumes the absorption of the delivered component from the interior surface of lung, into the lung tissue.

The present invention provides compositions and methods for the pulmonary administration of oligonucleotides. The compositions can contain carrier compounds, penetration enhancing agents, and/or transfection agents. As used herein, "carrier compound" refers to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioated oligonucleotide in hepatic tissue is reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid (Miyao *et al.*,



*Antisense Res. Dev.*, 1995, 5:115; Takakura *et al.*, *Antisense & Nucl. Acid Drug Dev.*, 1996, 6:177).

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, *etc.*, when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (*e.g.*, pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, *etc.*); fillers (*e.g.*, lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, *etc.*); lubricants (*e.g.*, magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, *etc.*); disintegrates (*e.g.*, starch, sodium starch glycolate, *etc.*); or wetting agents (*e.g.*, sodium lauryl sulphate, *etc.*).

In some embodiments, the present invention employs oligonucleotides for use in antisense modulation of the function of DNA or messenger RNA (mRNA) encoding a protein the modulation of which is desired, and ultimately to regulate the amount of such a protein. Hybridization of an antisense oligonucleotide with its mRNA target interferes with the normal role of mRNA and causes a modulation of its function in cells. The functions of mRNA to be interfered with include all vital functions such as translocation of the RNA to the site for protein translation, actual translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, turnover or degradation of the mRNA and possibly even independent catalytic activity which may be engaged in by the RNA. The overall effect of such interference with mRNA function is modulation of the expression of a protein, wherein "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of the protein. In the context of the present invention, inhibition is a suitable form of modulation of gene expression.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often suitable over native forms

because of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target and increased stability in the presence of nucleases.

An oligonucleotide is a polymer of repeating units generically known as nucleotides. An unmodified (naturally occurring) nucleotide has three components: (1) a nitrogenous *base* linked by one of its nitrogen atoms to (2) a 5-carbon cyclic *sugar* and (3) a *phosphate*, esterified to carbon 5 of the sugar. When incorporated into an oligonucleotide chain, the phosphate of a first nucleotide is also esterified to carbon 3 of the sugar of a second, adjacent nucleotide. The "backbone" of an unmodified oligonucleotide consists of (2) and (3), that is, sugars linked together by phosphodiester linkages between the carbon 5 (5') position of the sugar of a first nucleotide and the carbon 3 (3') position of a second, adjacent nucleotide. A "nucleoside" is the combination of (1) a nucleobase and (2) a sugar in the absence of (3) a phosphate moiety (Kornberg, A., *DNA Replication*, W.H. Freeman & Co., San Francisco, 1980, pages 4-7). The backbone of an oligonucleotide positions a series of bases in a specific order; the written representation of this series of bases, which is conventionally written in 5' to 3' order, is known as a nucleotide sequence.

Oligonucleotides may comprise nucleotide sequences sufficient in identity and number to effect specific hybridization with a particular nucleic acid. Such oligonucleotides which specifically hybridize to a portion of the sense strand of a gene are commonly described as "antisense." In the context of the invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleotides. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that an oligonucleotide need not be 100% complementary to its target DNA sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the

oligonucleotide to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a decrease or loss of function, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed.

Antisense oligonucleotides are commonly used as research reagents, diagnostic aids, and therapeutic agents. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes, for example to distinguish between the functions of various members of a biological pathway. This specific inhibitory effect has, therefore, been harnessed by those skilled in the art for research uses. The specificity and sensitivity of oligonucleotides is also harnessed by those of skill in the art for therapeutic uses. For example, the following U.S. patents demonstrate palliative, therapeutic and other methods utilizing antisense oligonucleotides. U. S. Patent No. 5,135,917 provides antisense oligonucleotides that inhibit human interleukin-1 receptor expression. U.S. Patent No. 5,098,890 is directed to antisense oligonucleotides complementary to the *c-myc* oncogene and antisense oligonucleotide therapies for certain cancerous conditions. U.S. Patent No. 5,087,617 provides methods for treating cancer patients with antisense oligonucleotides. U.S. Patent No. 5,166,195 provides oligonucleotide inhibitors of Human Immunodeficiency Virus (HIV). U.S. Patent No. 5,004,810 provides oligomers capable of hybridizing to herpes simplex virus Vmw65 mRNA and inhibiting replication. U.S. Patent No. 5,194,428 provides antisense oligonucleotides having antiviral activity against influenzavirus. U.S. Patent No. 4,806,463 provides antisense oligonucleotides and methods using them to inhibit HTLV-III replication. U.S. Patent No. 5,286,717 provides oligonucleotides having a complementary base sequence to a portion of an oncogene. U.S. Patent No. 5,276,019 and U.S. Patent No. 5,264,423 are directed to phosphorothioate oligonucleotide analogs used to prevent replication of foreign nucleic acids in cells. U.S. Patent No. 4,689,320 is directed to antisense oligonucleotides as antiviral agents specific to cytomegalovirus (CMV). U.S. Patent No. 5,098,890 provides oligonucleotides complementary to at least a portion of the mRNA transcript of the human *c-myc* gene. U.S. Patent No. 5,242,906 provides antisense oligonucleotides useful in the treatment of latent Epstein-Barr virus (EBV) infections. Other examples of antisense oligonucleotides are provided herein.

The oligonucleotides in accordance with this invention comprise from about 8 to about 30 nucleotides. Such oligonucleotides can comprise from about 15 to 25 nucleotides. As is known in the art, a nucleotide is a base-sugar combination suitably bound to an adjacent nucleotide through a phosphodiester, phosphorothioate or other covalent linkage. In the context  
5 of this invention, the term "oligonucleotide" includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides may be suitable over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target and  
10 increased stability in the presence of nucleases.

Oligonucleotides are also useful in determining the nature, function and potential relationship to body or disease states in animals of various genetic components of the body. Heretofore, the function of a gene has been chiefly examined by the construction of loss-of-function mutations in the gene (*i.e.*, "knock-out" mutations) in an animal (*e.g.*, a transgenic  
15 mouse). Such tasks are difficult, time-consuming and cannot be accomplished for genes essential to animal development since the "knock-out" mutation would produce a lethal phenotype. Moreover, the loss-of-function phenotype cannot be transiently introduced during a particular part of the animal's life cycle or disease state; the "knock-out" mutation is always present. "Antisense knockouts," that is, the selective modulation of expression of a gene by  
20 antisense oligonucleotides, rather than by direct genetic manipulation, overcomes these limitations (see, for example, Albert *et al.*, *Trends in Pharmacological Sciences*, 1994, 15:250). In addition, some genes produce a variety of mRNA transcripts as a result of processes such as alternative splicing; a "knock-out" mutation typically removes all forms of mRNA transcripts produced from such genes and thus cannot be used to examine the biological role of a particular  
25 mRNA transcript. By providing compositions and methods for the simple alimentary delivery of oligonucleotides and other nucleic acids, the present invention overcomes these and other shortcomings.

The present invention further encompasses compositions employing ribozymes. Synthetic RNA molecules and derivatives thereof that catalyze highly specific endoribonuclease  
30 activities are known as ribozymes. (See, generally, U.S. Patent No. 5,543,508 to Haseloff *et al.*, issued August 6, 1996, and U.S. Patent No. 5,545,729 to Goodchild *et al.*, issued August 13, 1996.) The cleavage reactions are catalyzed by the RNA molecules themselves. In naturally occurring RNA molecules, the sites of self-catalyzed cleavage are located within highly

conserved regions of RNA secondary structure (Buzayan *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 1986, 83, 8859; Forster *et al.*, *Cell*, 1987, 50, 9). Naturally occurring autocatalytic RNA molecules have been modified to generate ribozymes which can be targeted to a particular cellular or pathogenic RNA molecule with a high degree of specificity. Thus, ribozymes serve  
5 the same general purpose as antisense oligonucleotides (*i.e.*, modulation of expression of a specific gene) and, like oligonucleotides, are nucleic acids possessing significant portions of single-strandedness. That is, ribozymes have substantial chemical and functional identity with oligonucleotides and are thus considered to be equivalents for purposes of the present invention.

Other biologically active oligonucleotides may be formulated in the compositions of the  
10 invention and used for therapeutic, palliative or prophylactic purposes according to the methods of the invention. Such other biologically active oligonucleotides include, but are not limited to, antisense compounds including, *inter alia*, antisense oligonucleotides, antisense PNAs and ribozymes (described *supra*) and EGSs, as well as aptamers and molecular decoys (described *infra*).

15 Sequences that recruit RNase P are known as External Guide Sequences, hence the abbreviation "EGS." EGSs are antisense compounds that direct of an endogenous nuclease (RNase P) to a targeted nucleic acid (Forster *et al.*, *Science*, 1990, 249, 783; Guerrier-Takada *et al.*, *Proc. Natl. Acad. Sci. USA*, 1997, 94, 8468).

Antisense compounds may alternatively or additionally comprise a synthetic moiety  
20 having nuclease activity covalently linked to an oligonucleotide having an antisense sequence instead of relying upon recruitment of an endogenous nuclease. Synthetic moieties having nuclease activity include, but are not limited to, enzymatic RNAs (as in ribozymes), lanthanide ion complexes, and the like (Haseloff *et al.*, *Nature*, 1988, 334, 585; Baker *et al.*, *J. Am. Chem. Soc.*, 1997, 119, 8749).

25 Aptamers are single-stranded oligonucleotides that bind specific ligands via a mechanism other than Watson-Crick base pairing. Aptamers are typically targeted to, *e.g.*, a protein and are not designed to bind to a nucleic acid (Ellington *et al.*, *Nature*, 1990, 346, 818).

Molecular decoys are short double-stranded nucleic acids (including single-stranded nucleic acids designed to "fold back" on themselves) that mimic a site on a nucleic acid to which  
30 a factor, such as a protein, binds. Such decoys are expected to competitively inhibit the factor; that is, because the factor molecules are bound to an excess of the decoy, the concentration of factor bound to the cellular site corresponding to the decoy decreases, with resulting therapeutic,

palliative or prophylactic effects. Methods of identifying and constructing nucleic acid decoy molecules are described in, *e.g.*, U.S. Patent 5,716,780 to Edwards *et al.*

Another type of bioactive oligonucleotide is an RNA-DNA hybrid molecule that can direct gene conversion of an endogenous nucleic acid (Cole-Strauss *et al.*, *Science*, 1996, 273, 5 1386).

It has been discovered in accordance with the present invention that pulmonary administration of phosphodiester oligonucleotides is particularly advantageous. Specifically, it has been discovered in accordance with the present invention that the level of nuclease activity in lung tissue is sufficiently low to afford phosphodiester oligonucleotides longer lifetimes in lung  
10 tissue than was previously believed. Accordingly, contrary to conventional knowledge in the art (see, *e.g.*, Wu-Pong *et al.*, *Adv. Drug Delivery*, 1996, 19, 47), phosphodiester antisense oligonucleotides reside undegraded in the lung for a sufficiently long period of time to exert an antisense effect.

Therapeutic Considerations: In general, for therapeutic applications, a patient (*i.e.*, an  
15 animal, including a human, having, suspected of having, or predisposed to a disease or disorder) is administered one or more nucleic acids, including oligonucleotides, in accordance with the invention in doses ranging from 0.01 ug to 100 g per kg of body weight depending on the age of the patient and the severity of the disorder or disease state being treated. Further, the treatment regimen may last for a period of time which will vary depending upon the nature of the particular  
20 disease or disorder, its severity and the overall condition of the patient, and may extend from once daily to once every 20 years. In the context of the invention, the term "treatment" or "treatment regimen" is meant to encompass therapeutic, palliative and prophylactic modalities. Following treatment, the patient is monitored for changes in his/her condition and for alleviation of the symptoms of the disorder or disease state. The dosage of the nucleic acid may either be  
25 increased in the event the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the symptoms of the disorder or disease state is observed, or if the disorder or disease state has been ablated.

Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is  
30 effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides,

and can generally be estimated based on EC<sub>50</sub>s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 µg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. An optimal dosing schedule is used to deliver a therapeutically effective amount of the nucleic acid  
5 being administered via a particular mode of administration.

The term "therapeutically effective amount," for the purposes of the invention, refers to the amount of nucleic acid-containing formulation which is effective to achieve an intended purpose without undesirable side effects (such as toxicity, irritation or allergic response). Although individual needs may vary, determination of optimal ranges for effective amounts of  
10 formulations is within the skill of the art. Human doses can be extrapolated from animal studies (Katocs *et al.*, Chapter 27 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990). Generally, the dosage required to provide an effective amount of a formulation, which can be adjusted by one skilled in the art, will vary depending on the age, health, physical condition, weight, type and extent of the disease or disorder of the  
15 recipient, frequency of treatment, the nature of concurrent therapy (if any) and the nature and scope of the desired effect(s) (Nies *et al.*, Chapter 3 *In: Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.*, eds., McGraw-Hill, New York, NY, 1996).

As used herein, the term "high risk individual" is meant to refer to an individual for  
20 whom it has been determined, via, *e.g.*, individual or family history or genetic testing, has a significantly higher than normal probability of being susceptible to the onset or recurrence of a disease or disorder. As art of treatment regimen for a high risk individual, the individual can be prophylactically treated to prevent the onset or recurrence of the disease or disorder. The term "prophylactically effective amount" is meant to refer to an amount of a formulation which  
25 produces an effect observed as the prevention of the onset or recurrence of a disease or disorder. Prophylactically effective amounts of a formulation are typically determined by the effect they have compared to the effect observed when a second formulation lacking the active agent is administered to a similarly situated individual.

Following successful treatment, it may be desirable to have the patient undergo  
30 maintenance therapy to prevent the recurrence of the disease state, wherein the nucleic acid is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years. For example, in the case of in individual known or suspected of being prone to an autoimmune or inflammatory condition, prophylactic effects may

be achieved by administration of preventative doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years. In like fashion, an individual may be made less susceptible to an inflammatory condition that is expected to occur as a result of some medical treatment, *e.g.*, graft versus host disease resulting from the transplantation of cells, tissue  
5 or an organ into the individual.

The compositions of the present invention can include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

The pharmaceutical formulations, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical  
10 industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both.

Pharmaceutically Acceptable Salts: The compounds of the invention encompass any  
15 pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to "pharmaceutically acceptable salts" of the penetration enhancers and nucleic acids of the invention and prodrugs of such nucleic acids. "Pharmaceutically acceptable  
20 salts" are physiologically and pharmaceutically acceptable salts of the penetration enhancers and nucleic acids of the invention: *i.e.*, salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the oligonucleotide and nucleic acid compounds employed  
25 in the compositions of the present invention (*i.e.*, salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto).

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, ammonium, polyamines such as spermine and  
30 spermidine, and the like. Examples of suitable amines are chlorprocaine, choline, N,N'-dibenzylethylenediamine, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge *et al.*, "Pharmaceutical Salts," *J. of Pharma Sci.*, 1977, 66:1). The base addition salts of said acidic compounds are prepared by



contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention.

Oligonucleotide Prodrugs: The oligonucleotides of the invention may additionally or alternatively be prepared to be delivered in a "prodrug" form. The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (*i.e.*, drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin *et al.*, published December 9, 1993.

Oligonucleotide Deletion Derivatives: During the process of oligonucleotide synthesis, nucleoside monomers are attached to the chain one at a time in a repeated series of chemical reactions such as nucleoside monomer coupling, oxidation, capping and detritylation. The stepwise yield for each nucleoside addition is above 99%. That means that less than 1% of the sequence chain failed to the nucleoside monomer addition in each step as the total results of the incomplete coupling followed by the incomplete capping, detritylation and oxidation (Smith, *Anal. Chem.*, 1988, 60, 381A). All the shorter oligonucleotides, ranging from (n-1), (n-2), *etc.*, to 1-mers (nucleotides), are present as impurities in the n-mer oligonucleotide product. Among the impurities, (n-2)-mer and shorter oligonucleotide impurities are present in very small amounts and can be easily removed by chromatographic purification (Warren *et al.*, Chapter 9 *In: Methods in Molecular Biology, Vol. 26: Protocols for Oligonucleotide Conjugates*, Agrawal, S., Ed., 1994, Humana Press Inc., Totowa, NJ, pages 233-264). However, due to the lack of chromatographic selectivity and product yield, some (n-1)-mer impurities are still present in the full-length (*i.e.*, n-mer) oligonucleotide product after the purification process. The (n-1) portion consists of the mixture of all possible single base deletion sequences relative to the n-mer parent oligonucleotide. Such (n-1) impurities can be classified as terminal deletion or internal deletion sequences, depending upon the position of the missing base (*i.e.*, either at the 5' or 3' terminus or internally). When an oligonucleotide containing single base deletion sequence impurities is used as a drug (Crooke, *Hematologic Pathology*, 1995, 9, 59), the terminal deletion sequence impurities will bind to the same target mRNA as the full length sequence but with a slightly

lower affinity. Thus, to some extent, such impurities can be considered as part of the active drug component, and are thus considered to be bioequivalents for purposes of the present invention.

The compounds and method of the invention employ particles containing oligonucleotide therapeutics or diagnostics. The particles can be solid or liquid, and can be of  
5 respirable size: that is, particles of a size sufficiently small to pass through the mouth and larynx upon inhalation and into the bronchi and alveoli of the lungs. In general, particles ranging from about 5 to 20 microns in size are respirable and are expected to reach the bronchioles (Allen, *Secundum Artem*, Vol. 6, No. 3, on-line publication updated May 8, 1998, and available at <http://www.paddocklabs.com/secundum/secarndx.html>). It is greatly desirable to avoid particles  
10 of non-respirable size, as these tend to deposit in the throat and be swallowed, thus reducing the quantity of oligonucleotide reaching the lung.

Liquid pharmaceutical compositions of oligonucleotide can be prepared by combining the oligonucleotide with a suitable vehicle, for example sterile pyrogen free water, or saline solution. Other therapeutic compounds may optionally be included.

15 The present invention also contemplates the use of solid particulate compositions. Such compositions can comprise particles of oligonucleotide that are of respirable size. Such particles can be prepared by, for example, grinding dry oligonucleotide by conventional means, for example with a mortar and pestle, and then passing the resulting powder composition through a 400 mesh screen to segregate large particles and agglomerates. A solid particulate composition  
20 comprised of an active oligonucleotide can optionally contain a dispersant which serves to facilitate the formation of an aerosol, for example lactose.

In accordance with the methods of the present invention, oligonucleotide compositions are aerosolized. Aerosolization of liquid particles can be produced by any suitable means, such as with a nebulizer. See, for example, U.S. Patent No. 4,501,729. Nebulizers are commercially  
25 available devices which transform solutions or suspensions into a therapeutic aerosol mist either by means of acceleration of a compressed gas, typically air or oxygen, through a narrow venturi orifice or by means of ultrasonic agitation. Suitable nebulizers include those sold by Blairex® under the name PARI LC PLUS, PARI DURA-NEB 2000, PARI-BABY Size, PARI PRONEB Compressor with LC PLUS, PARI WALKHALER Compressor/Nebulizer System, PARI LC  
30 PLUS Reusable Nebulizer, and PARI LC Jet+® Nebulizer.

Exemplary formulations for use in nebulizers consist of an oligonucleotide in a liquid, such as sterile, pyrogen free water, or saline solution, wherein the oligonucleotide comprises up to about 40% w/w of the formulation. The oligonucleotide can comprise less than 20% w/w. If

desired, further additives such as preservatives (for example, methyl hydroxybenzoate) antioxidants, and flavoring agents can be added to the composition. Solid particles comprising an oligonucleotide can also be aerosolized using any solid particulate medicament aerosol generator known in the art. Such aerosol generators produce respirable particles, as described above, and further produce reproducible metered dose per unit volume of aerosol. Suitable solid particulate aerosol generators include insufflators and metered dose inhalers. Metered dose inhalers suitable for use in the art (along with the trade name, manufacturer and indication they are used for) and useful in the present invention include:

Delivery Device/Trade name/Manufacturer/Indication

10 Metered Dose Inhaler (MDI)

Alupent- Boehringer Ingelheim      Beta-adrenergic bronchodilator

Atrovent- Boehringer Ingelheim      Anticholinergic bronchodilator

Aerobid, Aerobid-M - Forest      Steriodal Anti-inflammatory

Beclovent, Beconase - Glaxo Wellcome      Steriodal Anti-inflammatory

15 Flovent - Glaxo Wellcome      Steriodal Anti-inflammatory

Ventolin - Glaxo Wellcome      Beta-adrenergic bronchodilator

Proventil - Key Pharm.      Beta-adrenergic bronchodilator

Maxair - 3M Pharm.      Beta-adrenergic bronchodilator

Azmacort - Rhone-Poulenc Rorer      Steriodal Anti-inflammatory

20 Tilade - Rhone-Poulenc Rorer      Anti-inflammatory (inhibits release of inflammatory mediators)

Intal - Rhone-Poulenc Rorer      Inhibits mast cell degranulation (Asthma)

Vanceril - Schering      Steriodal Anti-inflammatory

Tornalate - Dura Pharm.      Beta-adrenergic bronchodilator

25 Solutions for Nebulization

Alupent- Boehringer Ingelheim      Beta-adrenergic bronchodilator

Pulmozyme - Genetech      Recombinant human deoxyribonuclease I

Ventolin - Glaxo Wellcome      Beta-adrenergic bronchodilator

Tornalate - Dura Pharm.      Beta-adrenergic bronchodilator

30 Intal - Rhone-Poulenc Rorer      Inhibits mast cell degranulation (Asthma)

Capsules (powder) for inhalation      Ventolin - Glaxo Wellcome

(Rotocaps for use in Rotohaler device)      Beta-adrenergic bronchodilator

Powder for inhalation

Pulmicort - Astra USA

(Turbuhaler device) Steriodal Anti-inflammatory

Liquid or solid aerosols are produced at a rate of from about 10 to 150 liters per minute, or from about 30 to 150 liters per minute, or from about 60 liters per minute.

5 *Nebulization of oligonucleotides.*

Aqueous solutions of oligonucleotides are nebulized, and the resulting aerosol is delivered to an animal model (male CD-1 mice) via a nose-only inhalation system. In order to reach the bronchiolar and alveolar regions of the lung, the particle size is targeted for 1 to 5  $\mu\text{m}$ . Following single or multiple exposures, mice are evaluated for signs of toxicity and designated  
10 tissues are collected for assessment of organ-specific effects and the oligonucleotide concentrations. The male CD-1 mouse is chosen as the animal model for this study since considerable scientific data is available for this species.

Mice are given a 30 minute nose-only exposure of solutions of oligomeric compound having concentrations of either 10 or 100 mg/ml, with saline controls. Calculated lung doses are  
15 1.2 and 12 mg/kg, respectively. Animals are necropsied at 0 minutes (at the end of exposure), 2 hours, 8 hours, and 24 hours. Animals are generally assessed for their health, and more limited assessments are made of lung tolerability. Lung concentrations of oligonucleotide and oligonucleotide metabolites are performed by capillary gel electrophoresis (CGE) and distribution of oligonucleotide within lung tissue is determined immunohistologically.

20 The exposure systems used are designed to nebulize the test article solution or saline only. The exposure atmospheres are generated using PARI LC PLUS nebulizers (PARI Respiratory Equipment, Inc, Richmond, VA). Filtered compressed air is used as the air supply. Airflow rates are set and maintained at levels required to assure a consistent aerosol generation and maintain animal health. Empty ports within the generation chamber provide locations for  
25 obtaining samples for gravimetric and particle size determination or analysis.

Atmosphere concentration is determined both gravimetrically (development phase) and by analytical measurements (animal exposure). Glass fiber filters (Gelman #66075, Gelman sciences, Ann Arbor, MI) are placed into in-line filter holders. Airflow rates are regulated to sample a known volume of test atmosphere. Immediately after sampling, the filters are collected  
30 and the mass concentration calculated. The filter samples are then processed to extract and analyze the test material deposited on the filter. Analytical measurements are used to calculate the inhaled dose. Samples are collected during each exposure in which animals are placed in the chambers.

Particle size is measured with a Mercer style cascade impactor (Chen *et al.*, *Fundam. Appl. Toxicol.*, 1989, 13, 429). The effective cut-off diameters for the impactor can range from 4.8 microns to 0.30 microns. Particle size is measured for each oligonucleotide tested, following the first and last exposure. The Mass Median Aerodynamic Diameter (MMAD) for the three  
 5 oligonucleotides can range from 2.72 to 3.26 and the Geometric Standard Deviation (GSD) can range from 2.44 to 2.46.

Animals are exposed in nose-only exposure units similar to the design described by Cannon et al (1983), *Amer. Ind. Hyg. Assoc.* 44(12) 923-928. "Open" type restraint tubes are used to aid in the ability of the animals to thermoregulate and elimination of excreta. The  
 10 pulmonary dose is calculated based on the following equation:

$$\text{Pulmonary Dose} = \frac{\text{RMV} \times \text{Concentration} \times \text{Time} \times \text{Deposition Factor}}{\text{Body Weight}}$$

wherein:

RMV = respiratory minute volume, assumed to be 0.03 l/min for a 30 gram mouse

15 Concentration = chamber concentration based on analytical methods

Time = exposure time in minutes

Deposition Factor = fraction that remains in lung, assumed to be 10% with a particle size of 2 to 3 micrometers.

Body Weight = mean body weight in grams (30 grams is used as the average)

20

Based on this equation, and the data obtained following filter analysis, the estimated pulmonary dose for the low, mid, and high dose groups is approximately 0.8, 1.5 and 3.2 mg/kg, respectively.

The concentration of each oligonucleotide and its metabolites can be determined in  
 25 tissue samples of lung, liver, kidney and spleen.

### **Example 9: Compositions and Methods for the Non-Parenteral Delivery of Nucleic Acids**

Enhanced bioavailability of oligonucleotides and other nucleic acids is achieved via the non-parenteral administration of the compositions and methods of the present invention. The  
 30 term "bioavailability" refers to a measurement of what portion of an administered drug reaches the circulatory system when a non-parenteral mode of administration is used to introduce the drug into an animal. The term is used for drugs whose efficacy is related to the blood concentration achieved, even if the drug's ultimate site of action is intracellular (van Berge-

Henegouwen et al., *Gastroenterol.*, 1977, 73, 300). Traditionally, bioavailability studies determine the degree of intestinal absorption of a drug by measuring the change in peripheral blood levels of the drug after an oral dose (DiSanto, Chapter 76 In: *Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages 1451-1458).

5 The area under the curve (AUC<sub>0</sub>) is divided by the area under the curve after an intravenous (i.v.) dose (AUC<sub>iv</sub>) and the quotient is used to calculate the fraction of drug absorbed. This approach cannot be used, however, with compounds which have a large "first pass clearance," i.e., compounds for which hepatic uptake is so rapid that only a fraction of the absorbed material enters the peripheral blood. For such compounds, other methods must be used to determine the

10 absolute bioavailability (van Berge-Henegouwen et al., *Gastroenterol.*, 1977, 73, 300). With regards to oligonucleotides, studies suggest that they are rapidly eliminated from plasma and accumulate mainly in the liver and kidney after i.v. administration (Miyao et al., *Antisense Res. Dev.*, 1995, 5, 115; Takakura et al., *Antisense & Nucl. Acid Drug Dev.*, 1996, 6, 177).

Another "first pass effect" that applies to orally administered drugs is degradation due

15 to the action of gastric acid and various digestive enzymes. Furthermore, the entry of many high molecular weight active agents (such as peptides, proteins and oligonucleotides) and some conventional and/or low molecular weight drugs (e.g., insulin, vasopressin, leucine enkephalin, etc.) through mucosal routes (such as oral, pulmonary, buccal, rectal, transdermal, vaginal and ocular) to the bloodstream is frequently obstructed by poor transport across epithelial cells and

20 concurrent metabolism during transport. This type of degradative metabolism is known for oligonucleotides and nucleic acids. For example, phosphodiesterases are known to cleave the phosphodiester linkages of oligonucleotides and many other modified linkages present in synthetic oligonucleotides and nucleic acids.

One means of ameliorating first pass clearance effects is to increase the dose of

25 administered drug, thereby compensating for proportion of drug lost to first pass clearance. Although this may be readily achieved with i.v. administration by, for example, simply providing more of the drug to an animal, other factors influence the bioavailability of drugs administered via non-parenteral means. For example, a drug may be enzymatically or chemically degraded in the alimentary canal or blood stream and/or may be impermeable or semipermeable to various

30 mucosal membranes.

It has now been found that oligonucleotides can be introduced effectively into animals via non-parenteral means through coadministration of "mucosal penetration enhancers," also known as "absorption enhancers" or simply as "penetration enhancers". These are substances

which facilitate the transport of a drug across mucous membrane(s) associated with the desired mode of administration.

A "pharmaceutically acceptable" component of a formulation of the invention is one which, when used together with excipients, diluents, stabilizers, preservatives and other ingredients are appropriate to the nature, composition and mode of administration of a formulation. Accordingly it is desired to select penetration enhancers which facilitate the uptake of oligonucleotides, without interfering with the activity of the oligonucleotides and in a manner such that the same can be introduced into the body of an animal without unacceptable side-effects such as toxicity, irritation or allergic response.

10 The present invention provides compositions comprising one or more pharmaceutically acceptable penetration enhancers, and methods of using such compositions, which result in the improved bioavailability of nucleic acids administered via non-parenteral modes of administration. Heretofore, certain penetration enhancers have been used to improve the bioavailability of certain drugs. See Muranishi, Crit. Rev. Ther. Drug Carrier Systems, 1990, 7, 1 and Lee et al., Crit. Rev. Ther. Drug Carrier Systems, 1991, 8, 91. However, it is generally viewed to be the case that effectiveness of such penetration enhancers is unpredictable. Therefore, it has been surprisingly found that the uptake and delivery of oligonucleotides, relatively complex molecules which are known to be difficult to administer to animals and man, can be greatly improved even when administered by non-parenteral means through the use of a number of different classes of penetration enhancers.

The effective non-parenteral use and administration of compositions of the present invention involves consideration of a number of different aspects about drug therapy. One important consideration when using the compositions and methods of the present invention is the mode of administration of the pharmaceutical composition containing the therapeutic oligonucleotide or other nucleic acid. Administration typically is either parenteral or non-parenteral. Non-parenteral modes of administration include, but are not limited to, buccal, sublingual, endoscopic, oral, rectal, transdermal, topical, nasal, intratracheal, pulmonary, urethral, vaginal, and ocular. When administered by such non-parenteral modes the methods and composition of the present invention can deliver drug both locally and systemically as desired.

30 A second consideration of importance when using the compositions and methods of the present invention is the use and nature of penetration enhancers and carriers. Penetration enhancers facilitate the transport of drug molecules, for example, oligonucleotides and other nucleic acids, across mucosal and other epithelial cell membranes. Penetration enhancers

include, but are not limited to, members of molecular classes such as surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactant molecules. Carriers are inert molecules that may be included in the compositions of the present invention to interfere with processes that lead to reduction in the levels of bioavailable nucleic acid or oligonucleotide drug.

5           A third consideration of importance to the compositions and methods of the present invention is the nature of oligonucleotide or other nucleic acid used. Oligonucleotides of the present invention may be, but are not limited to, those nucleic acids bearing modified linkages, modified nucleobases, or modified sugars, and chimeric nucleic acids.

10           A fourth consideration of importance in the present invention is the nature of the composition. Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions (including microemulsions and creams), and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids. The compositions of the present invention may be formulated into any of many possible dosage  
15 forms such as, but not limited to, tablets, capsules, liquid syrups, soft gels, suppositories, and enemas.

          A fifth consideration of importance to the compositions and methods of the present invention is the means by which such compositions may be administered. Thus the dose, method of administration or application, and the use of additives are all worthy of consideration in this  
20 regard. Further, the methods and compositions of the present invention may be used to ameliorate a variety of diseases via local or systemic treatment. Such local or systemic treatment may be accomplished using the methods and compositions of the present invention via modes of administration that include, but are not limited to, buccal, sublingual, endoscopic, oral, rectal, transdermal, topical, nasal, pulmonary, urethral, vaginal, and ocular modes.

25           A sixth consideration of importance to the compositions and methods of the present invention is their applicability to bioequivalents of oligonucleotides and other nucleic acids such as, but not limited to, oligonucleotide prodrugs, deletion derivatives, conjugates, aptamers, and ribozymes.

          The present invention provides compositions and methods for local and systemic  
30 delivery of one or more nucleic acids to an animal via non-parenteral administration. For purposes of the invention, the term "animal" is meant to encompass humans as well as other mammals, as well as reptiles, fish, amphibians, and birds. The term "non-parenteral delivery" refers to the administration, directly or otherwise, of the drug via a non-invasive procedure



which typically does not entail the use of a syringe and needle. Non-parenteral administration may be, but is not limited to, delivery of the drug via the alimentary canal or via transdermal, topical, nasal, pulmonary, urethral, vaginal or ocular routes. The term "alimentary canal" refers to the tubular passage in an animal that functions in the digestion and absorption of food and the  
5 elimination of food residue, which runs from the mouth to the anus, and any and all of its portions or segments, e.g., the oral cavity, the esophagus, the stomach, the small and large intestines and the colon, as well as compound portions thereof such as, e.g., the gastro-intestinal tract. Thus, the term "alimentary delivery" encompasses several routes of administration including, but not limited to, oral, rectal, endoscopic and sublingual/buccal administration. A  
10 common requirement for these modes of administration is absorption over some portion or all of the alimentary tract and a need for efficient mucosal penetration of the nucleic acid(s) so administered.

In addition, iontophoresis (transfer of ionic solutes through biological membranes under the influence of an electric field) (Lee et al., Critical Reviews in Therapeutic Drug Carrier  
15 Systems, 1991, p. 163), phonophoresis or sonophoresis (use of ultrasound to enhance the absorption of various therapeutic agents across biological membranes, notably the skin and the cornea) (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 166), and optimization of vehicle characteristics relative to dose deposition and retention at the site of administration (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 168)  
20 may be useful methods for enhancing the transport of drugs across mucosal sites in accordance with the present invention.

Delivery of a drug via the oral mucosa, as in the case of buccal and sublingual administration, has several desirable features, including, in many instances, a more rapid rise in plasma concentration of the drug than via oral delivery (Harvey, Chapter 35 In: Remington=s  
25 Pharmaceutical Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, page 711). Furthermore, because venous drainage from the mouth is to the superior vena cava, this route also bypasses rapid first-pass metabolism by the liver. Both of these features contribute to the sublingual route being the mode of choice for drugs like nitroglycerin (Benet et al., Chapter 1  
In: Goodman & Gilman=s The Pharmacological Basis of Therapeutics, 9th Ed., Hardman et al.,  
30 eds., McGraw-Hill, New York, NY, 1996, page 7).

Endoscopy may be used for drug delivery directly to an interior portion of the alimentary tract. For example, endoscopic retrograde cystopancreatography (ERCP) takes advantage of extended gastroscopy and permits selective access to the biliary tract and the

pancreatic duct (Hirahata et al., *Gan To Kagaku Ryoho*, 1992, 19(10 Suppl.), 1591). Pharmaceutical compositions, including liposomal formulations, can be delivered directly into portions of the alimentary canal, such as, e.g., the duodenum (Somogyi et al., *Pharm. Res.*, 1995, 12, 149) or the gastric submucosa (Akamo et al., *Japanese J. Cancer Res.*, 1994, 85, 652) via  
5 endoscopic means. Gastric lavage devices (Inoue et al., *Artif. Organs*, 1997, 21, 28) and percutaneous endoscopic feeding devices (Pennington et al., *Ailment Pharmacol. Ther.*, 1995, 9, 471) can also be used for direct alimentary delivery of pharmaceutical compositions.

Drugs administered by the oral route can often be alternatively administered by the lower enteral route, i.e., through the anus into the rectum or lower intestine. Rectal  
10 suppositories, retention enemas or rectal catheters can be used for this purpose and may be preferred when patient compliance might otherwise be difficult to achieve (e.g., in pediatric and geriatric applications, or when the patient is vomiting or unconscious). Rectal administration can result in more prompt and higher blood levels than the oral route. (Harvey, Chapter 35 In: *Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton,  
15 PA, 1990, page 711). Because about 50% of the drug that is absorbed from the rectum will bypass the liver, administration by this route significantly reduces the potential for first-pass metabolism (Benet et al., Chapter 1 In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al., eds., McGraw-Hill, New York, NY, 1996).

One method of non-parenteral administration for most drugs is oral delivery. This is  
20 typically the most convenient route for access to the systemic circulation. Absorption from the alimentary canal is governed by factors that are generally applicable, e.g., surface area for absorption, blood flow to the site of absorption, the physical state of the drug and its concentration at the site of absorption (Benet et al., Chapter 1 In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al., eds., McGraw-Hill, New York,  
25 NY, 1996, pages 5-7). A significant factor which may limit the oral bioavailability of a drug is the degree of "first pass effects." For example, some substances have such a rapid hepatic uptake that only a fraction of the material absorbed enters the peripheral blood (Van Berge-Henegouwen et al., *Gastroenterology*, 1977, 73:300). The compositions and methods of the invention circumvent, at least partially, such first pass effects by providing improved uptake of  
30 nucleic acids by, e.g., causing the hepatic uptake system to become saturated and allowing a significant portion of the nucleic acid so administered to reach the peripheral circulation.

Topical administration is often chosen when local delivery of a drug is desired at, or immediately adjacent to the point of application of the drug composition or formulation.

Although occasionally enough drug is absorbed into the systemic circulation to cause systemic effects, topical routes generally are not effective for systemic therapy. Three general types of topical routes of administration are recognized, topical administration of a drug composition to mucous membranes, skin or eyes.

5        Drugs that are applied to the mucous membranes produce primarily local effects. This route of administration includes application of drug compositions to mucous membranes of the conjunctiva, nasopharynx, oropharynx, vagina, colon, urethra, and urinary bladder. Absorption of drugs occurs rapidly through mucous membranes and is an effective route for localized therapy and, on occasion, for systemic therapy.

10        Transdermal drug delivery is a valuable route for the administration of lipid soluble therapeutics. It has been recognized that the dermis is more permeable than the epidermis and therefore absorption of drugs is much more rapid through abraded, burned or denuded skin. Inflammation and other physiologic conditions that increase blood flow to the skin also enhance absorption via the transdermal route. Absorption by this route may be enhanced via the use of an  
15    oily vehicle (inunction) or through the use of penetration enhancers. Hydration of the skin and the use of controlled release topical patches are also effective ways to administer drugs via the transdermal route. This route provides a means to deliver the drug for both systemic and local therapy.

      Ocular delivery of drugs is especially useful for the local treatment of eye infections or  
20    abnormalities. The drug is typically administered via instillation and absorption of the drug occurs through the cornea. Corneal infection or trauma may thus result in more rapid absorption. Ophthalmic delivery systems that provide prolonged duration of action (e.g., suspensions and ointments) and ocular inserts that provide continuous delivery of low amounts of drugs are useful additions to ophthalmic therapy. The ocular delivery of drugs results in predominantly  
25    local effects. Systemic absorption that results from drainage via the nasolachrial canal is limited and few systemic side effects are typically observed.

      The present invention employs various penetration enhancers in order to effect transport of oligonucleotides and other nucleic acids across mucosal and epithelial membranes. Penetration enhancers may be classified as belonging to one of five broad categories -  
30    surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 92). Each of these classes is discussed in more detail in the following paragraphs. Carrier substances (or simply "carriers"),

which reduce first pass effects by, e.g., saturating the hepatic uptake system, are also herein described.

In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the alimentary mucosa and other epithelial membranes is enhanced. In addition to bile salts and fatty acids, surfactants include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92); and perfluorochemical emulsions, such as FC-43 (Takahashi et al., *J. Pharm. Pharmacol.*, 1988, 40, 252).

Fatty acids and their derivatives which act as penetration enhancers and may be used in compositions of the present invention include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprates, tricaprates, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glyceryl 1-monocaprates, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines and mono- and di-glycerides thereof and/or physiologically acceptable salts thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1; El-Hariri et al., *J. Pharm. Pharmacol.*, 1992, 44, 651).

A variety of bile salts also function as penetration enhancers to facilitate the uptake and bioavailability of drugs. The physiological roles of bile include the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 In: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al., eds., McGraw-Hill, New York, NY, 1996, pages 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus, the term "bile salt" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucolic acid (sodium glucolate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (CDCA, sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium

glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Swinyard, Chapter 39 In: Remington's Pharmaceutical Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages 782-783; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1; 5 Yamamoto et al., J. Pharm. Exp. Ther., 1992, 263, 25; Yamashita et al., J. Pharm. Sci., 1990, 79, 579).

In a particular embodiment, penetration enhancers useful in the present invention are mixtures of penetration enhancing compounds. For example, a suitable penetration enhancer is a mixture of UDCA (and/or CDCA) with capric and/or lauric acids or salts thereof e.g. sodium. 10 Such mixtures are useful for enhancing the delivery of biologically active substances across mucosal membranes, in particular intestinal mucosa. Suitable penetration enhancer mixtures comprise about 5-95% of bile acid or salt(s) UDCA and/or CDCA with 5-95% capric and/or lauric acid. Particularly suitable are mixtures of the sodium salts of UDCA, capric acid and lauric acid in a ratio of about 1:2:2 respectively.

15 Chelating agents, as used in connection with the present invention, can be defined to be compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of oligonucleotides through the alimentary and other mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases 20 require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, J. Chromatogr., 1993, 618, 315). Chelating agents of the invention include, but are not limited to, disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, lauric acid and N-amino acyl derivatives of beta-diketones (enamines)(Lee et al., Critical Reviews in Therapeutic Drug 25 Carrier Systems, 1991, page 92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1; Buur et al., J. Control Rel., 1990, 14, 43).

As used herein, non-chelating non-surfactant penetration enhancers may be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary and other mucosal 30 membranes (Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1). This class of penetration enhancers includes, but is not limited to, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac

sodium, indomethacin and phenylbutazone (Yamashita et al., J. Pharm. Pharmacol., 1987, 39, 621).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi et al, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo et al., PCT Application WO 97/30731), can be used.

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (i.e., does not possess biological activity per se) but is recognized as a nucleic acid by in vivo processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'isothiocyano-stilbene-2,2'-disulfonic acid (Miyao et al., Antisense Res. Dev., 1995, 5, 115; Takakura et al., Antisense & Nucl. Acid Drug Dev., 1996, 6, 177).

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate,

sodium acetate, etc.); disintegrants (e.g., starch, sodium starch glycolate, EXPLOTAB); and wetting agents (e.g., sodium lauryl sulphate, etc.).

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art established usage  
5 levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically active materials such as, for example, antipruritics, astringents, local anesthetics or anti inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the composition of present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers.  
10 However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention.

The present invention employs oligonucleotides for use in antisense modulation of the function of DNA or messenger RNA (mRNA) encoding a protein the modulation of which is desired, and ultimately to regulate the amount of such a protein. Hybridization of an antisense  
15 oligonucleotide with its mRNA target interferes with the normal role of mRNA and causes a modulation of its function in cells. The functions of mRNA to be interfered with include all vital functions such as translocation of the RNA to the site for protein translation, actual translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, turnover or degradation of the mRNA and possibly even independent catalytic activity which may be  
20 engaged in by the RNA. The overall effect of such interference with mRNA function is modulation of the expression of a protein, wherein "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of the protein. In the context of the present invention, inhibition is one form of modulation of gene expression.

The present invention also includes compositions employing oligonucleotides that are  
25 substantially chirally pure with regard to particular positions within the oligonucleotides. Examples of substantially chirally pure oligonucleotides include, but are not limited to, those having phosphorothioate linkages that are at least 75% Sp or Rp (Cook et al., U.S. Patent No. 5,587,361) and those having substantially chirally pure (Sp or Rp) alkylphosphonate, phosphoamidate or phosphotriester linkages (Cook, U.S. Patents Nos. 5,212,295 and 5,521,302).

30 Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, ammonium, polyamines such as spermine and spermidine, and the like. Examples of suitable amines are chlorprocaine, choline, N,N'

dibenzylethylenediamine, diethanolamine, dicyclohexylamine, ethylenediamine, N methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," J. of Pharma Sci., 1977, 66:1). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids. The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, liquid syrups, soft gels, suppositories, and enemas.

Pharmaceutically acceptable organic or inorganic carrier substances suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and formulations containing liposomes. While basically similar in nature these formulations vary in the components and the consistency



of the final product. The know-how on the preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the present invention.

The compositions of the present invention may be prepared and formulated as  
5 emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1  $\mu\text{m}$  in diameter. (Idson, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 199; Rosoff, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 245;  
10 Block, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 2, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 335; Higuchi et al., in "Remington's *Pharmaceutical Sciences*," Mack Publishing Co., Easton, PA, 1985, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water in oil (w/o) or  
15 of the oil in water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the resulting composition is called a water in oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil in water (o/w) emulsion.

20 Emulsions may contain additional components in addition to the dispersed phases and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the  
25 case of oil in water in oil (o/w/o) and water in oil in water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

30 Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the

case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 285; Idson, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group into: nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 335; Idson, Id., p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethyl cellulose and carboxypropyl cellulose), and synthetic polymers (for example, carbomers, cellulose ethers, 5 and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations 10 often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated 15 hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, 20 Inc., New York, NY, 1988, p. 199). Emulsion formulations for oral delivery have been very widely used because of reasons of ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 245; Idson, Id., p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations 25 are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, 30 Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been

described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: Controlled Release of Drugs: Polymers and Aggregate Systems, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a  
5 combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, 1985, p. 271).

10 The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in Pharmaceutical Dosage Forms: Disperse Systems, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 245; Block, Id., p. 335). Compared to conventional emulsions, microemulsions offer the advantage of  
15 solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310),  
20 hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film  
25 because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not  
30 limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, 1993, 5 13, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385; Ho et al., *J. Pharm. Sci.*, 1996, 10 85, 138). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will 15 facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve 20 the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 92). Each of these classes has been 25 discussed above.

There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. Further advantages 30 are that liposomes obtained from natural phospholipids are biocompatible and biodegradable, liposomes can incorporate a wide range of water and lipid soluble drugs, liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds.,

Marcel Dekker, Inc., New York, NY, 1988, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes. Liposomes can be administered orally and in aerosols and topical applications.

Surfactants find wide application in formulations such as emulsions (including  
5 microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman,  
10 Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene  
15 glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/ propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

20 If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic  
25 surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

30 If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 285).

In one embodiment of the invention, a nucleic acid is administered via the rectal mode.

5 In particular, compositions for rectal administration include solutions (enemas and suppositories) and emulsions. Rectal suppositories for adults are usually tapered at one or both ends and typically weigh about 2 g each, with infant rectal suppositories typically weighing about one-half as much, when the usual base, cocoa butter, is used (Block, Chapter 87 In: *Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990).

10 The use of absorption-promoting adjuvants is known in the art for the modification of the barrier function of the rectal membrane and has been reviewed (Nishihata and Rytting, *Advanced Drug Delivery Reviews*, 1997, 28, 205). Absorption-promoting adjuvants have shown promising effects on the performance of formulations of poorly absorbed drugs such as moderately large water-soluble drugs and peptides. Enamine derivatives of amino acids have  
15 exhibited absorption promoting properties but the mechanism by which they increase rectal absorption is unclear. Compounds such as chelating agents, and sulfhydryl depleters have been shown to increase the rectal absorption of drugs through the paracellular route as well as the transcellular route. Salicylate and its derivatives also increase absorption of drugs administered via the rectal route via both paracellular and transcellular paths. Fatty acids show properties  
20 similar to salicylates when enhancing rectal absorption of drugs. Lectin is also known to increase rectal absorption of drugs via induction of microvillus infusion.

In One embodiment of the invention, one or more nucleic acids are administered via oral delivery.

Compositions for oral administration include powders or granules, suspensions or  
25 solutions in water or non-aqueous media, capsules, sachets, troches, tablets or SECs (soft elastic capsules or "caplets"). Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids, carrier substances or binders may be desirably added to such formulations. A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine, the active  
30 ingredients in a free flowing form such as a powder or granules, optionally mixed with a binder (PVP or gums such as tragacanth, acacia, carrageenan), lubricant (e.g. stearates such as magnesium stearate), glidant (talc, colloidal silica dioxide), inert diluent, preservative, surface active or dispersing agent. Suitable binders/disintegrants include EMDEX (dextrate),

PRECIROL (triglyceride), PEG, and AVICEL (cellulose). Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredients therein.

5           The use of such formulations has the effect of delivering the nucleic acid to the alimentary canal for exposure to the mucosa thereof. Accordingly, the formulation can contain an enteric material effective in protecting the nucleic acid from pH extremes of the stomach, or in releasing the nucleic acid over time to optimize the delivery thereof to a particular mucosal site. Enteric materials for acid-resistant tablets, capsules and caplets are known in the art and  
10 typically include acetate phthalate, propylene glycol, sorbitan monoleate, cellulose acetate phthalate (CAP), cellulose acetate trimellitate and hydroxy propyl methyl cellulose phthalate (HPMCP). Enteric materials may be incorporated within the dosage form or may be a coating substantially covering the entire surface of tablets, capsules or caplets. Enteric materials may also be accompanied by plasticizers which impart flexible resiliency to the material for resisting  
15 fracturing, for example during tablet curing or aging. Plasticizers are known in the art and typically include diethyl phthalate (DEP), triacetin, dibutyl sebacate (DBS), dibutyl phthalate (DBP) and triethyl citrate (TEC).

Various methods for producing formulations for alimentary delivery are well known in the art. See, generally, Nairn, Chapter 83; Block, Chapter 87; Rudnic et al., Chapter 89; Porter, Chapter  
20 90; and Longer et al., Chapter 91 In: Remington's Pharmaceutical Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990. The compositions of this invention can be converted in a known manner into the customary formulations, such as tablets, coated tablets, pills, granules, capsules, aerosols, syrups, emulsions, suspensions and solutions, using inert, non toxic, pharmaceutically suitable excipients or solvents. The therapeutically active compound  
25 should in each case be present here in a concentration of about 0.5% to about 95% by weight of the total mixture, that is to say in amounts which are sufficient to achieve the stated dosage range. Compositions may be formulated in a conventional manner using additional pharmaceutically acceptable carriers or excipients as appropriate. Thus, the composition may be prepared by conventional means with carriers or excipients such as binding agents (e.g.,  
30 pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulfate). Tablets may be coated by methods well known in



the art. The preparations may also contain flavoring, coloring and/or sweetening agents as appropriate.

Capsules used for oral delivery may include formulations that are well known in the art. Further, multicompartiment hard capsules with control release properties as described by Digenis et al., U.S. Patent No. 5,672,359, and water permeable capsules with a multi-stage drug delivery system as described by Amidon et al., U.S. Patent No. 5,674,530 may also be used to formulate the compositions of the present invention.

The formulation of pharmaceutical compositions and their subsequent administration is believed to be within the skill of those in the art. Specific comments regarding the present invention are presented below.

In general, for therapeutic applications, a patient (i.e., an animal, including a human) having or predisposed to a disease or disorder is administered one or more nucleic acids, including oligonucleotides, in accordance with the invention in a pharmaceutically acceptable carrier in doses ranging from 0.01 ug to 100 g per kg of body weight depending on the age of the patient and the severity of the disorder or disease state being treated. Further, the treatment regimen may last for a period of time which will vary depending upon the nature of the particular disease or disorder, its severity and the overall condition of the patient, and may extend from once daily to once every 20 years. In the context of the invention, the term "treatment regimen" is meant to encompass therapeutic, palliative and prophylactic modalities. Following treatment, the patient is monitored for changes in his/her condition and for alleviation of the symptoms of the disorder or disease state. The dosage of the nucleic acid may either be increased if the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the symptoms of the disorder or disease state is observed, or if the disorder or disease state has been abated.

Formulations for non-parenteral administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic carrier substances suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like. The formulations can be sterilized and, if desired, mixed with

auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation. Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for  
5 example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

The pharmaceutical formulations, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients  
10 with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

A number of bioequivalents of oligonucleotides and other nucleic acids may also be employed in accordance with the present invention. The invention therefore, also encompasses  
15 oligonucleotide and nucleic acid equivalents such as, but not limited to, prodrugs of oligonucleotides and nucleic acids, deletion derivatives, conjugates of oligonucleotides, aptamers, and ribozymes.

The methods and compositions of the present invention also encompass the myriad deletion oligonucleotides, both internal and terminal deletion oligonucleotides, that are  
20 synthesized during the process of solid-phase manufacture of oligonucleotides for such deletion sequences are for all practical purposes bioequivalents. Synthetic RNA molecules and their derivatives that possess specific catalytic activities are known as ribozymes and are also considered bioequivalents of oligonucleotides for the purposes of the methods and compositions of the present invention. Also considered bioequivalents of oligonucleotides, for the purposes of  
25 the methods and compositions of the present invention, are peptide nucleic acids (PNAs) and aptamers (see, generally, Ellington et al., Nature, 1990, 346, 818; U.S. Patent 5,523,389 (Ecker et al., June 4, 1996)).

The name aptamer has been coined by Ellington and Szostak (Nature, 1990, 346, 818) for nucleic acid molecules that fit and therefore bind with significant specificity to non-nucleic  
30 acid ligands such as peptides, proteins and small molecules such as drugs and dyes. Because of these specific ligand binding properties, nucleic acids and oligonucleotides that may be classified as aptamers may be readily purified or isolated via affinity chromatography using columns that bear immobilized ligand. Aptamers may be nucleic acids that are relatively short to those that

are as large as a few hundred nucleotides. For example, Ellington and Szostak have reported the discovery of RNA aptamers that are 155 nucleotides long and that bind dyes such as Cibacron Blue and Reactive Blue 4 (Ellington and Szostak, *Nature*, 1990, 346, 818) with very good selectivity. While RNA molecules were first referred to as aptamers, the term as used in the present invention refers to any nucleic acid or oligonucleotide that exhibits specific binding to small molecule ligands including, but not limited to, DNA, RNA, DNA derivatives and conjugates, RNA derivatives and conjugates, modified oligonucleotides, chimeric oligonucleotides, and gapmers.

The invention is drawn to the non-parenteral administration of a nucleic acid, such as an oligonucleotide, having biological activity, to an animal. By "having biological activity," it is meant that the nucleic acid functions to modulate the expression of one or more genes in an animal as reflected in either absolute function of the gene (such as ribozyme activity) or by production of proteins coded by such genes. In the context of this invention, "to modulate" means to either effect an increase (stimulate) or a decrease (inhibit) in the expression of a gene. Such modulation can be achieved by, for example, an antisense oligonucleotide by a variety of mechanisms known in the art, including but not limited to transcriptional arrest; effects on RNA processing (capping, polyadenylation and splicing) and transportation; enhancement or reduction of cellular degradation of the target nucleic acid; and translational arrest (Crooke et al., *Exp. Opin. Ther. Patents*, 1996, 6, 1).

In an animal other than a human, the compositions and methods of the invention can be used to study the function of one or more genes in the animal. For example, antisense oligonucleotides have been systemically administered to rats in order to study the role of the N-methyl-D-aspartate receptor in neuronal death, to mice in order to investigate the biological role of protein kinase C- $\alpha$ , and to rats in order to examine the role of the neuropeptide Y1 receptor in anxiety (Wahlestedt et al., *Nature*, 1993, 363, 260; Dean et al., *Proc. Natl. Acad. Sci. U.S.A.*, 1994, 91, 11762; and Wahlestedt et al., *Science*, 1993, 259, 528, respectively). In instances where complex families of related proteins are being investigated, "antisense knockouts" (i.e., inhibition of a gene by systemic administration of antisense oligonucleotides) may represent the most accurate means for examining a specific member of the family (see, generally, Albert et al., *Trends Pharmacol. Sci.*, 1994, 15, 250).

As stated, the compositions and methods of the invention are useful therapeutically, i.e., to provide therapeutic, palliative or prophylactic relief to an animal, including a human, having or suspected of having or of being susceptible to, a disease or disorder that is treatable in whole

or in part with one or more nucleic acids. The term "disease or disorder" (1) includes any abnormal condition of an organism or part, especially as a consequence of infection, inherent weakness, environmental stress, that impairs normal physiological functioning; (2) excludes pregnancy per se but not autoimmune and other diseases associated with pregnancy; and (3) includes cancers and tumors. The term "having or suspected of having or of being susceptible to" indicates that the subject animal has been determined to be, or is suspected of being, at increased risk, relative to the general population of such animals, of developing a particular disease or disorder as herein defined. For example, a subject animal could have a personal and/or family medical history that includes frequent occurrences of a particular disease or disorder. As another example, a subject animal could have had such a susceptibility determined by genetic screening according to techniques known in the art (see, e.g., U.S. Congress, Office of Technology Assessment, Chapter 5 In: Genetic Monitoring and Screening in the Workplace, OTA-BA-455, U.S. Government Printing Office, Washington, D.C., 1990, pages 75-99). The term "a disease or disorder that is treatable in whole or in part with one or more nucleic acids" refers to a disease or disorder, as herein defined, (1) the management, modulation or treatment thereof, and/or (2) therapeutic, palliative and/or prophylactic relief therefrom, can be provided via the administration of more nucleic acids. In One embodiment, such a disease or disorder is treatable in whole or in part with an antisense oligonucleotide.

*Evaluation of Formulations By In Situ Perfusion of Rat Ileum*

Methods: In order to evaluate the formulations, in situ perfusion of rat ileum is performed essentially according to the procedure of Komiya et al. (Int. J. Pharmaceut., 1980, 4:249). Specifically, male Sprague Dawley rats weighing 250-300 g are used. After overnight fasting, the rats are anesthetized with 5% pentobarbital (50 mg/kg) by intraperitoneal injection. After a midline abdominal incision is made, the small intestine is taken out and ileum section located. An incision is made at each end of a 20 cm ileum segment. The ileum segment is laid out in a uniform multiple-S arrangement with 3 bends. The luminal contents of the section are flushed with buffer. A 10 cm piece of silicon rubber tubing is inserted into the intestinal lumen at each incision and ligated with 3-0 silk suture. The proximal end tubing is connected to a 30 mL syringe containing oligonucleotide solution. The formulation is perfused through the intestinal segment by using Sage model 365 syringe pump at 0.125 mL/min. The outflow solution is collected in a 2 mL centrifuge tube over 5 min intervals for 80 mins. At the end of perfusion study, an aliquot of 0.3 mL blood sample is collected from the portal vein.

The oligonucleotide concentration in the solution before and after passing through a 20 cm ileum segment is analyzed by high pressure liquid chromatography (HPLC) while the plasma samples are analyzed by capillary electrophoresis (CE). In most cases, tritium labeled oligomeric compound is used as a tracer and the radioactivity of solution measured by a liquid scintillation counter. The amount of the drug absorbed from the ileum is calculated by dividing the concentration from the average of last six outflow samples (steady state) to that of the inflow sample.

*Evaluation of the Bioavailability of Oligonucleotide from Formulations Following In Vivo (Intrajejunum) Instillation*

10 In order to evaluate the absolute oral bioavailability of an oligomeric compound in formulations containing various penetration enhancers, *in vivo* intrajejunum instillation can be performed with the following formulations:

Formulation 4a: First, 100 mg CDCA was transferred to a 5 ml volumetric flask containing about 3 ml of buffer. The flask was shaken until the CDCA was completely dissolved. Next, 200 mg sodium caprate and 200 mg sodium laurate were added to the solution, and the flask was shaken until all of the solid material was completely dissolved. Then, 0.5 ml of ISIS 2302 stock solution (200 mg/ml) was added to the solution. Finally, the volume of the solution was adjusted to 5 ml with buffer.

Formulation 4b: First, 200 mg sodium caprate and 200 mg sodium laurate were transferred to a 5 ml volumetric flask containing about 3 ml of buffer. Then, 100 mg of UDCA was added and the flask was shaken until the UDCA was completely dissolved. Then, 0.5 ml of ISIS 2302 stock solution (200 mg/ml) was added to the solution. Finally, the volume of the solution was adjusted to 5 ml with buffer.

Formulation 4c: A microemulsion of ISIS 2302 was prepared essentially according to the procedures of Panayiotis (Pharm. Res., 1984, 11:1385). An aliquot of 0.6 ml of ISIS 2302 stock solution (200 mg/ml) was transferred to a 30 ml beaker containing 1.0 ml of Tween 80 (Sigma Chemical Company St. Louis, MO). Next, a mixture of 6.3 ml of Captex 355 (Abitec Corp., Janesville, WI) and 2.1 ml of Capmul MCM (Abitec Corp.) was added to the beaker. The resultant solution was stirred until a clear solution was formed.

30 Methods: Precannulated Sprague-Dawley rats weighing 250-300 g were used. After overnight fasting, the rats were anesthetized with 5% pentobarbital (50 mg/kg) by intraperitoneal injection. After a midline abdominal incision was made, the small intestine was pulled out and injection site was located (2 cm after the ligament of Treitz). An aliquot of 1.0 mL drug solution

was then injected via a 27 gauge needle. The intestine was put back to the body carefully. Muscle was then surgically closed and skin was clipped after injection. Three hundred  $\mu\text{L}$  of blood was collected from a cannula at each sampling time point. The rats were sacrificed in the CO<sub>2</sub> chamber 24 hours after dosing. Livers and kidneys were excised and stored at -80°C until  
5 analysis. Radioactivity of plasma and tissue samples were measured. Liver and kidney were also analyzed for oligonucleotide content by capillary gel electrophoresis (CGE).

#### *Preparation of Microemulsion Formulations*

In order to evaluate the bioavailability of oligonucleotide microemulsions the following microemulsion formulations of ISIS 2302 were prepared:

10 Formulation 5a: A microemulsion of ISIS 2302 was prepared essentially according to the procedures of Panayiotis (Pharm. Res., 1984, 11:1385). An aliquot of 0.6 ml of ISIS 2302 stock solution (200 mg/ml) was transferred to a 30 ml beaker containing 1.0 ml of Tween 80 (Sigma Chemical Company St. Louis, MO). Next, a mixture of 6.3 ml of Captex 355 (Abitec Corp., Janesville, WI) and 2.1 ml of Capmul MCM (Abitec Corp., Janesville, WI) was added to  
15 the beaker. The resultant solution was stirred until a clear solution was formed.

Formulation 5b: A water-in-oil microemulsion of ISIS 2302 was prepared essentially by adding the oil phase to the aqueous phase with adequate mixing. The aqueous phase was prepared by mixing 1 ml of a 100 mg/ml solution of ISIS 2302 and 1 ml of Tween 80 (Sigma Chemical Company St. Louis, MO). The oil phase was prepared by mixing 3 parts of Captex  
20 355 (Abitec Corp., Janesville, WI) and 1 part of Capmul MCM (Abitec Corp., Janesville, WI). The oil phase was added to the aqueous phase with adequate stirring until the resultant mixture was a clear solution.

#### *Preparation of Water-in-Oil (w/o) Cream Formulations*

In order to evaluate the bioavailability of oligonucleotide emulsions the water-in-oil  
25 cream formulations of ISIS 2302 were prepared as follows:

Formulation 6a1: A water-in-oil cream formulation of ISIS 2302 was prepared by first preparing the two phases. A 2 ml aliquot of the ISIS 2302 stock solution (100 mg/ml) was mixed with 2 ml water in a 10 ml beaker and warmed to 70°C. A mixture of 1 gram of Grill 3 (Croda, U.S.), 3 ml Captex 355 (Abitec Corp., Janesville, WI) and 3 ml of Labrasol (Gattefosse,  
30 France) was prepared in a 30 ml beaker and this mixture was also warmed to 70°C. The aqueous solution of oligonucleotide was then poured slowly into the oil phase with vigorous mixing. Upon cooling to ambient temperature the desired water-in-oil cream formulation of oligonucleotide (~20 mg/mL) was obtained.

Formulation 6a2: A water-in-oil cream formulation of ISIS 2302 was prepared by first preparing the two phases. A 1.5 ml aliquot of the ISIS 2302 stock solution (200 mg/ml) was transferred to a 10 ml beaker and warmed to 70°C. In a 30 ml beaker were placed 0.5 gram of Grill 3 (Croda, U.S.), 1.5 ml Captex 355 (Abitec Corp., Janesville, WI), and 1.5 ml of Labrasol 5 (Gattefosse, France) and this mixture also warmed to 70°C. The aqueous solution of oligonucleotide was then poured slowly into the oil phase with vigorous mixing. Upon cooling to ambient temperature the desired water-in-oil cream formulation oligonucleotide (~60 mg/mL) was obtained.

#### *Water-in-Oil Cream Formulations*

10 Formulation 7a: An oil-in-water cream formulation of ISIS 2302 was prepared by first preparing the two phases. A 0.5 ml aliquot of the ISIS 2302 stock solution (200 mg/ml) was mixed with 0.5 ml of Tween 80 (Sigma Chemical Company St. Louis, MO) and 1.8 ml water in a 30 ml beaker and warmed to about 70°C. In a 10 ml beaker were placed 100 mg. of Grill 3 (Croda, U.S.), 1 ml Captex 355 (Abitec Corp., Janesville, WI), and 1 ml of Labrasol (Gattefosse, 15 France) and this mixture also warmed to about 70°C. The oil phase was then poured into the aqueous solution of oligonucleotide with vigorous mixing. Upon cooling to ambient temperature the desired oil-in-water cream formulation was obtained.

Various natural bile salts, and their synthetic derivatives act as penetration enhancers. The physiological roles of bile include the facilitation of dispersion and absorption of lipids and 20 fat-soluble vitamins (Brunton, Chapter 38 In: Goodman & Gilman=s The Pharmacological Basis of Therapeutics, 9th Ed., Goodman et al., eds., McGraw-Hill, New York, NY, 1996, pages 934-935). Bile salt derived penetration enhancers include, for example, cholic acid, cholalic acid or 3a,7a,12a-trihydroxy-5b-cholan-24-oic acid (or its pharmaceutically acceptable sodium salt); deoxycholic acid, desoxycholic acid, 5b-cholan-24-oic acid-3a,12a-diol, 7-deoxycholic acid or 25 3a,12a-dihydroxy-5b-cholan-24-oic acid (sodium deoxycholate); glycocholic acid, (N-[3a,7a,12a-trihydroxy-24-oxocholan-24-yl]glycine or 3a,7a,12a-trihydroxy-5b-cholan-24-oic acid N-[carboxymethyl]amide or sodium glycocholate); glycodeoxycholic acid, (5b-cholan-24-oic acid N-[carboxymethyl]amide-3a,12a-diol), 3a,12a-dihydroxy-5b-cholan-24-oic acid N-[carboxymethyl]amide, N-[3a,12a-dihydroxy-24-oxocholan-24-yl]glycine or glycodesoxycholic 30 acid (sodium glycodeoxycholate); taurocholic acid, (5b-cholan-24-oic acid N-[2-sulfoethyl]amide-3a,7a,12a-triol), 3a,7a,12a-trihydroxy-5b-cholan-24-oic acid N-[2-sulfoethyl]amide or 2-[(3a,7a,12a-trihydroxy-24-oxo-5b-cholan-24-yl)amino] ethanesulfonic acid (sodium taurocholate); taurodeoxycholic acid, (3a,12a-dihydroxy-5b-cholan-2-oic acid N[2-

5 sulfoethyl]amide or 2-[(3a,12a-dihydroxy-24-oxo-5b-cholan-24-yl)-amino]ethanesulfonic acid, or sodium taurodeoxycholate, or sodium taurodesoxycholate); chenodeoxycholic acid (chenodiol, chenodesoxycholic acid, 5b-cholanic acid-3a,7a-diol, 3a,7a-dihydroxy-5b-cholanic acid, or sodium chenodeoxycholate, or CDCA); ursodeoxycholic acid, (5b-cholan-24-oic acid-  
 5 3a,7b-diol, 7b-hydroxylithocholic acid or 3a,7b-dihydroxy-5b-cholan-24-oic acid, or UDCA); sodium taurodihydro-fusidate (STDHF); and sodium glycodihydrofusidate (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Swinyard, Chapter 39 In: Remington's Pharmaceutical Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages 782-783).

#### 10 Sources of Penetration Enhancers

##### A. FATTY ACIDS AND DERIVATIVES

Compound Name	Abbreviation	Supplier
Capric acid, Na salt	caprate	SigmaH
Lauric acid, Na salt	laurate	Sigma

##### 15 BILE SALTS AND DERIVATIVES

Compound Name	Abbreviation	Supplier
Cholic acid, Na salt	CA	Sigma
Glycholic acid, Na salt	GCA	Sigma
Glycodeoxycholic acid, Na Salt	GDCA	Sigma
20 Taurocholic acid, Na salt	TCA	Sigma
Taurodeoxycholic acid, Na salt	TDCA	Sigma
Chenodeoxycholic acid, Na salt	CDCA	Sigma
Ursodeoxycholic acid	UDCA	Aldrich

25 Formulation 9a: An emulsion formulation of ISIS 2302 was prepared by first preparing the two phases. A 0.5 ml aliquot of the ISIS 2302 stock solution (200 mg/ml) was mixed with 1.0 ml of the mixture of penetration enhancers (chenodeoxycholic acid sodium salt, sodium laurate and sodium caprate) and 0.5 ml water, and warmed to about 70°C (aqueous phase). A separate mixture of 500 mg. of Grill 3 (Croda International Plc., East Yorkshire, U.K.), 1.25 ml  
 30 Captex 355 (Abitec Corp., Janesville, WI), and 1.25 ml of Labrasol (Gattefosse Corp., Westwood, N.J.) was also prepared and warmed to about 70°C (oil phase). The aqueous phase was then transferred to the oil phase with vigorous mixing to afford the desired emulsion



concentration of 20 mg/ml ISIS 2302. The aqueous phase of emulsion contained 2% CDCA, 4% Sodium laurate, and 4% Sodium caprate.

Formulation 9b: Aliquots of the stock solution of ISIS 2302 (200 mg/ml) and mixture of penetration enhancers were mixed to afford a solution formulation comprising ISIS 2302 at a concentration of 20 mg/ml and a final concentration of 2% CDCA, 4% Sodium laurate and 4% Sodium caprate.

Formulation 9c: An emulsion formulation of ISIS 2302 was prepared by first preparing the two phases. A 0.5 ml aliquot of the ISIS 2302 stock solution (200 mg/ml) was mixed with 1.0 ml of the mixture of penetration enhancers (ursodeoxycholic acid sodium salt, sodium laurate and sodium caprate) and 0.5 ml water, and warmed to about 70°C. A separate mixture of 500 mg. of Grill 3 (Croda, U.S.), 1.25 ml Captex 355 (Abitec Corp., Janesville, WI), and 1.25 ml of Labrasol (Gattefosse, France) was also prepared and warmed to about 70°C. The aqueous phase was then transferred to the oil phase with vigorous mixing to afford the desired emulsion concentration of 20 mg/ml ISIS 2302. The aqueous phase of emulsion contained 2% UDCA, 4% Sodium laurate, and 4% Sodium caprate.

Formulation 9d: Aliquots of the stock solution of ISIS 2302 (200 mg/ml) and mixture of penetration enhancers were mixed to afford a solution formulation comprising ISIS 2302 at a concentration of 20 mg/ml and a final concentration of 2% UDCA, 4% Sodium laurate, and 4% Sodium caprate.

#### *Evaluation of Emulsion Formulations Containing Penetration Enhancers*

In order to determine the ability of penetration enhancers to improve the absorption and delivery of oligonucleotide drugs the emulsion formulations of Example 9 were administered via intrajejunum instillation and the blood concentrations of the oligonucleotide and AUC(0-3h) were measured.

Methods: Sprague-Dawley rats weighing 250-300 g were used. After overnight fasting, the rats were anesthetized with 5% pentobarbital (50 mg/kg) by intraperitoneal injection. After a midline abdominal incision was made, the small intestine was pulled out and injection site was located (2 cm after the ligament of Treitz). An aliquot of 0.5 mL drug formulation was then injected via a 27 gauge needle. The intestine was put back into the body carefully. The incision portion was covered with a wet gauze. 300 µL of blood was collected by a 27 gauge needle from the femoral vein at each sampling time point.

Formulations: Two emulsions (Formulations 9a and 9c) were evaluated at a dosage level of 10 mg/rat. The delivery of ISIS 2302 using these emulsions (Formulations 9a and 9c) formulated

with a combination of penetration enhancers was compared to the performance of solutions (Formulations 9b and 9d, respectively) of ISIS 2302 that were formulated with the same combination and concentration of penetration enhancers.

*Preparation of Enema Formulations*

5 To evaluate the delivery and mucosal penetration of oligonucleotides into the colon following rectal delivery, the following formulations were prepared. Solution and emulsion formulations of ISIS 2302 were prepared. Additives used in the formulations included saline, hydroxypropyl methyl cellulose (HPMC), carrageenan, Vitamin E  $\alpha$ -tocopheryl polyethylene glycol 1000 succinate (TPGS), Tween 80 and sorbitol.

10 Formulation 12a: A solution of ISIS 2302 was prepared by mixing 5 ml ISIS 2302 stock solution (100 mg/mL) with 95 ml sterile saline to have a final concentration of 5 mg/ml.

Formulation 12b: The solution was prepared by mixing 7.5 ml ISIS 2302 stock solution (100 mg/mL) with 142.5 ml hydroxypropyl methyl cellulose solution (HPMC) to have final concentrations of ISIS 2302 5 mg/ml and HPMC 15 mg/ml (1.5%). The HPMC solution was  
15 prepared by dissolving 2.25 g HPMC in 30 ml of 80°C water and Q.S. to 142.5 mL with cold water.

Formulation 12c: The solution was prepared by mixing 7.5 ml ISIS 2302 stock solution (100 mg/ml) with 142.5 ml carrageenan/Vitamin E TPGS solution to have a final concentration of ISIS 2302 5 mg/ml, carrageenan 10 mg/ml (1%), and Vitamin E TPGS 25 mg/ml (2.5%).  
20 The carrageenan/Vitamin E TPGS solution was prepared by dissolving 1.5g carrageenan and 3.75 g Vitamin E TPGS in 142.5 ml water. The solution was then heated to 60°C to form a gel and cooled down to room temperature before the addition of ISIS 2302 stock solution.

Formulation 12d: A water-in-oil emulsion (w/o) of ISIS 2302 was prepared following the general methods in Examples 5 and 6. The emulsion containing 5 mg/ml of ISIS 2302.

25 Formulation 12e: The solution was prepared by mixing 6.0 ml ISIS 2302 stock solution (100 mg/ml) with 0.6 ml tween 80, and 113.4 ml HPMC solution to have final concentrations of ISIS 2302 5 mg/ml, tween 80 50  $\mu$ l/ml (0.5%), and HPMC 7.5 mg/ml (0.75%). The HPMC solution was prepared by dissolving 0.9 g HPMC in 60 ml of 80°C water and Q.S. to 113.4 ml with cool water.

30 Formulation 12f: The solution was prepared by mixing 6.0 ml ISIS 2302 stock solution (100 mg/ml) with 6 g sorbitol, and 108 ml HPMC solution to have final concentrations of ISIS 2302 5 mg/ml, sorbitol 50 mg/ml (5%), and HPMC 6.3 mg/ml (0.63%). The HPMC solution

was prepared by dissolving 0.75 g HPMC in 50 ml of 80°C water and Q.S. to 108 ml with cool water.

Formulation 12g: A solution of ISIS 2302 was prepared by mixing 5 ml ISIS 2302 stock solution (100 mg/ml) with 95 ml water to have a final concentration of 5 mg/ml

#### 5 *Evaluation of Enema Formulations for Local Delivery of Oligonucleotide*

Formulations of oligonucleotide were evaluated via rectal administration as enemas to laboratory beagle dogs.

Methods: Following a period of overnight fasting, test dogs were first administered a cleansing enema and then dosed with a sample of the test formulation. The enema formulation was applied via a Foley catheter and held for a period of 1 h. In order to assess colonic tissue delivery and uptake of oligonucleotide, colon tissue biopsies were performed on the test animal, 3h and 24 h after dosing. Tissue samples were processed and the amount of oligonucleotide present in the tissue assessed by capillary gel electrophoresis (CGE) and immunohistochemical (IHC) analyses.

The present disclosure thus provides for localized delivery of oligonucleotides and other small nucleic acids to the lower portion of the G.I. tract. Such delivery can be via means of an enema using a solution comprising an effective concentration of oligonucleotide. Alternatively, suppositories comprising oligonucleotides suspended in an agent that disperses its contents when exposed to the physical and/or chemical conditions of the colon. In addition to HPMC, One dispersing agent for localized colonic delivery of oligonucleotides is cocoa butter.

Immunohistochemical (IHC) analyses were used to confirm and extend these results by determining the histopathology and cellular localization of rectally administered oligonucleotides in dogs. Biopsy samples were taken 10 to 20 centimeters from (i.e., approximately 18, 20, 21 and 22 cm from) the dorsal side of the colon and evaluated for the distribution of phosphorothioate oligodeoxynucleotide (P=S ODN) ISIS-2302 three hours after rectal administration. The biopsy samples were fixed in 10% neutral buffered formalin for 24 hours and transferred to 70% for storage. The tissues were embedded in paraffin and sectioned at 5  $\mu$ m for immunohistochemical detection of P=S ODN. The affinity purified antibody used for this work, 2E1-B5, is a mouse IgG1 (Berkeley Antibody Company, Richmond, CA) which specifically recognizes P=S ODN.

Tissues were deparaffinized and pre-treated with proteinase K (Dako Corp., Carpinteria, CA) for 10 minutes at room temperature prior to incubation in the primary antibody. The antibody was detected with donkey anti mouse f(ab')<sub>2</sub> IgG conjugated to horseradish

peroxidase (Jackson Laboratories, West Grove, PA) and diaminobenzidine (DAB, Dako Corp.) was used as a substrate. All slides were stained on the Dako automated immuno-stainer.

Staining of the P=S ODN is seen in the nucleus of the surface epithelial layer at the tips of the colonic villi in all of the biopsies. The staining is strongest in the 20-22 cm samples and some staining is seen at the luminal surface of the epithelium, which is most likely associated with mucinous material in the colon.

*Composition of and Preparation of Oral Dosage Formulations*

The following oral dosage formulations of oligonucleotides were prepared as follows.

Oral Dosage Formulation a: ISIS 2302 with penetration enhancers (CDCA, SC, SL) and excipient precirol.

Oligonucleotide (ISIS 2302) was passed through a 60 mesh screen, 12.4 g of which was then mixed with 10 g sodium chenodeoxycholate (CDCA), 20 g of sodium caprate (SC), 20 g sodium laurate (SL) and 47.5 g precirol (WL 2155 ATO, prescreened on 60 mesh). The powder was then placed in a plastic bag, mixed thoroughly and then sifted through a 20 mesh screen. Powder blend was then compressed into tablets at slight weight overage using round flat-faced tooling. The resulting 1100 " 50 mg tablets contained 124 mg oligonucleotide (as is by weight), 100 mg CDCA, 200 mg SL, 200 mg SC, and 476 mg precirol. Resultant tablets may be used as is (core tablets) or may be enteric film coated as described below under "Enteric Coating".

Oral Dosage Formulation b: ISIS 2302 with penetration enhancers (CDCA, SC, SL) and excipient (PEG).

Oligonucleotide (ISIS 2302) was passed through a 60 mesh screen and 9.3 g of which was mixed with 7.5 g sodium chenodeoxycholate (CDCA), 15 g of sodium caprate (SC), 15 g sodium laurate (SL) and 35.7 g polyethyleneglycol (20,000 mw PEG, prescreened 20 mesh). The powder was then placed in a plastic bag, mixed thoroughly and sifted through 20 mesh screen. Powder blend was then compressed into tablets at slight weight overage using round flat-faced tooling. The resulting 1100 " 50 mg tablets contained 124 mg oligonucleotide (as is by weight), 100 mg CDCA, 200 mg SL, 200 mg SC, and 476 mg PEG. Resultant tablets may be used as is (core tablets) or may be enteric film coated as described below under "Enteric Coating".

Oral Dosage Formulation c: ISIS 15839 with penetration enhancers (CDCA, SC, SL) and excipient (PEG).

ISIS 15839 is a phosphorothioate isosequence "hemimer" derivative of ISIS 2302 having "C" residues have 5-methylcytosine (m5c) bases and further comprising a 2'-

methoxyethoxy modification (other residues are 2'-deoxy). ISIS 15839 is described in co-pending U.S. Patent application Serial No. 09/062,416, filed April 17, 1998, hereby incorporated by reference.

ISIS 15839 was passed through a 60 mesh screen and 2.323 g of which was mixed with  
5 2.0 g sodium chenodeoxycholate (CDCA), 4.0 g of sodium caprate (SC), 4.0 g sodium laurate (SL) and 9.523 g polyethyleneglycol (20,000 mw PEG, prescreened 20 mesh). The powder was then sifted through a 20 mesh screen and placed in a plastic bag and mixed thoroughly. Powder blend was then compressed into tablets at slight weight overage using 12 mm round tooling. The resulting 728.4 “ 10 mg tablets contained 77.44 mg oligonucleotide, 66.67 mg CDCA, 133.4 mg  
10 SL, 133.4 mg SC, and 317.5 mg PEG. Resultant tablets may be used as is (core tablets) may be enteric film coated as described below under “Enteric Coating”.

Oral Dosage Formulation d: ISIS 2302 with penetration enhancers (CDCA, SC, SL) without excipient.

Oligonucleotide ISIS 2302 was passed through a 60 mesh screen and 3.72 g of which  
15 was mixed with 3 g sodium chenodeoxycholate (CDCA), 6 g of sodium caprate (SC) and 6 g sodium laurate (SL). The powder was sifted through a 20 mesh screen and placed in plastic bag and mixed thoroughly. This powder blend was then compressed into tablets at slight weight overage using 12 mm diameter tooling. The resulting 624 “ 10 mg tablets contained 124 mg oligonucleotide (as is by weight), 100 mg CDCA, 200 mg SL and 200 mg SC. Resultant tablets  
20 may be used as is (core tablets) or may be enteric film coated as described below under “Enteric Coating”.

#### Enteric Coating (EC)

A cellulose acetate phthalate (CAP) enteric coating solution was prepared by slowly adding 7.0 g of CAP powder to 90.0 g stirred acetone. Before dissolving, 3.0 g diethyl phthalate  
25 was added and the solution covered with aluminum foil and continued to stir until dissolution was complete after approximately 30 minutes.

Core tablets were coated by hand by dipping into a stirred CAP solution using vacuum tubing to hold the tablet. As the coating dried the tablets were inverted and redipped to effect completion of a single coat over entire surface. This method may be repeated to impart an  
30 adequate enteric film coverage of 2 to 5% weight gain and, depending on tablet size and configuration, to allow for a uniform coat thickness and performance quality.

#### *Evaluation of Oral Dosage Formulations*

The tablet formulations were evaluated according to the following methods.

In Vitro: In order to evaluate the integrity of the enteric film coat, tablets were placed in 500 mL aqueous 0.1 N HCL acid solution (pH 1.5) using USP method II (paddles) at 150 rpm and 37°C for up to 1 hour. Filtered samples were periodically taken and analyzed for presence of oligonucleotide as described below. Absence of oligonucleotide and visual inspection verified enteric coat integrity. Tablets were then placed into 500 mL of 0.2 M phosphate buffer solution, pH 6.5, to evaluate the rate at which oligonucleotides were released out of the tablets. Dissolution was monitored at regular time intervals by analyzing sample filtrate using UV light at 260 nm. This analysis was suitable for formulations devoid of interfering components (i.e., dissolved excipients capable of absorbing at 260 nm). Alternatively, samples may be analyzed by any of various separation methods (e.g., HPLC).

In Vivo: In order to measure the bioavailability of oligonucleotides from tablet formulations, tablets were administered orally (p.o.) to healthy beagle dogs of ~12 kg average weight at an approximate dose of 15 mg/kg. Blood samples were taken at regular time intervals and plasma harvested then subsequently analyzed for presence of oligonucleotide by either high pressure liquid chromatography (HPLC) for screening purposes or capillary gel electrophoresis (CGE) for purposes of confirmation and/or quantitation. Baseline pharmacokinetic intravenous (i.v.) data were obtained by administration of sterile drug solution (2 mg/kg) by slow i.v. push via antecubital vein followed by phlebotomies and analysis as described above.

#### *Use of Other Animal Models to Evaluate Formulations*

In order to further evaluate the bioavailability of the formulations of the invention, various animal models are used. For example, rectal formulations are tested in rats essentially according to the method of Aungst et al. (Pharm. Res., 1988, 5:305) or in rabbits essentially according to the methods of Buur et al. (J. Control Rel., 1990, 14:43) and Yamamoto et al. (J. Pharmacol. Exper. Therapeutics, 1992, 263:25).

The formulations of the invention are further evaluated in larger animals for optimization of the penetration enhancer (PE) systems in terms of, e.g., concentration and temporal effects on the absorption of oligonucleotides (ODN). Dogs will be "ported" with intestinal access catheters through which formulated drug formulations (solutions or suspensions) may be introduced into various areas of the gut. Target areas include the proximal jejunum and distal ileum or the ileocecal junction. These respective areas provide for ideal assessment of the systemic oligonucleotide bioavailability and for local tissue (e.g., colonic) absorption. This latter objective is assessed on the basis of both tissue biopsy drug levels and/or inferred by the presence of drug in the plasma. In addition to ported dogs, naive dogs will be

used for the assessment of formulations given by conventional routes, e.g., oral administration for oral dosage forms, rectal administration for enema or suppository formulations, etc. Dogs are dosed at 10 mg/kg of oligonucleotides, which are appropriately labeled as necessary, and blood samples are collected and evaluated for the presence and concentration of oligonucleotides. The absolute bioavailability is calculated and, if necessary, animals are sacrificed and tissue samples are collected and analyzed.\

#### **Example 10: Compositions and Methods for Topical Delivery of Oligonucleotides**

The present invention provides methods and compositions for delivery of nucleic acids, particularly oligonucleotides, to the epidermis and/or dermis of an animal to increase the bioavailability of the nucleic acid therein. As used herein, the term "bioavailability" refers to the amount of the administered drug therapy (in this case the oligonucleotide) that reaches and acts upon its target. The term is used for drugs whose efficacy is measured relative to the concentration in the blood even though the ultimate site of action of the drug might be outside the blood, e.g., intracellular (see van Berge-Henegouwen et al., *Gastroenterology*, 1977, 73, 300).

The compositions and methods of the invention may be used to provide prophylactic, palliative or therapeutic relief from a disease or disorder that is treatable in whole or in part with one or more nucleic acids. In One embodiment, such a disease or disorder is treatable in whole or in part via topical administration of an antisense oligonucleotide to an animal having such a disease or disorder.

The term "skin," as used herein, refers to the epidermis and/or dermis of an animal. Mammalian skin consists of two major, distinct layers. The outer layer of the skin is called the epidermis. The epidermis is comprised of the stratum corneum, the stratum granulosum, the stratum spinosum, and the stratum basale, with the stratum corneum being at the surface of the skin and the stratum basale being the deepest portion of the epidermis. The epidermis is between 50  $\mu$ m and 0.2 mm thick, depending on its location on the body.

Beneath the epidermis is the dermis, which is significantly thicker than the epidermis. The dermis is primarily composed of collagen in the form of fibrous bundles. The collagenous bundles provide support for, inter alia, blood vessels, lymph capillaries, glands, nerve endings and immunologically active cells.

One of the major functions of the skin as an organ is to regulate the entry of substances into the body. The principal permeability barrier of the skin is provided by the stratum

corneum, which is formed from many layers of cells in various states of differentiation. The spaces between cells in the stratum corneum is filled with different lipids arranged in lattice-like formations which provide seals to further enhance the skin's permeability barrier.

The permeability barrier provided by the skin is such that it is largely impermeable to  
5 molecules having molecular weight greater than about 750 Da. For larger molecules to cross the skin's permeability barrier, mechanisms other than normal osmosis must be used. Consequently, there is a need for compositions and methods to facilitate the transport of nucleic acids through the skin's permeability barrier to the epidermis and the dermis.

Several factors determine the permeability of the skin to administered agents. These  
10 factors include the characteristics of the treated skin, the characteristics of the delivery agent, interactions between both the drug and delivery agent and the drug and skin, the dosage of the drug applied, the form of treatment, and the post treatment regimen. To selectively target the epidermis and dermis, it is sometimes possible to formulate a composition that comprises one or more penetration enhancers that will enable penetration of the drug to a preselected stratum.

15 One method for the delivery of biologically active substances to the skin is topical administration. Topical administration can be used as the route of administration when local delivery of a drug is desired at, or immediately adjacent to, the point of application of the drug composition or formulation. Three general types of topical routes of administration include administration of a drug composition to mucous membranes, skin or eyes.

20 Transdermal drug delivery is a valuable route for the administration of lipid soluble therapeutics. The dermis is more permeable than the epidermis and therefore absorption is much more rapid through abraded, burned or denuded skin. Inflammation and other physiologic conditions that increase blood flow to the skin also enhance transdermal adsorption. Absorption via this route may be enhanced by the use of an oily vehicle (inunction) or through the use of one  
25 or more penetration enhancers. Other effective ways to deliver drugs via the transdermal route include hydration of the skin and the use of controlled release topical patches. The transdermal route provides a potentially effective means to deliver a drug for systemic and/or local therapy.

In addition, iontophoresis (transfer of ionic solutes through biological membranes under the influence of an electric field) (Lee et al., Critical Reviews in Therapeutic Drug Carrier  
30 Systems, 1991, p. 163), phonophoresis or sonophoresis (use of ultrasound to enhance the absorption of various therapeutic agents across biological membranes, notably the skin and the cornea) (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 166), and optimization of vehicle characteristics relative to dose deposition and retention at the site of



administration (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 168) may be useful methods for enhancing the transport of drugs across mucosal sites in accordance with compositions and methods of the present invention.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product. The know-how on the preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the present invention.

The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1  $\mu\text{m}$  in diameter. (Idson, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 199; Rosoff, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 245; Block in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume

2, p. 335; Higuchi et al., in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, 1985, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water in oil (w/o) or of the oil in water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the resulting composition is called a water in oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil in water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil in water in oil (o/w/o) and water in oil in water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 285; Idson, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting

surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group into: nonionic, anionic, cationic and amphoteric (Rieger, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 285).

5 Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These  
10 include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations  
15 and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 335; Idson, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 199).

20 Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethyl cellulose and carboxypropyl cellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that  
25 stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion  
30 formulations include methylparaben, propylparaben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, boric acid and phenoxyethanol. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated

hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

Preservatives used in any oligonucleotide formulation will preferably have a broad spectrum of antimicrobial activity and be compatible with highly negatively charged  
5 oligonucleotides at neutral pH. To determine suitable preservatives, oligonucleotides were incubated with various preservatives in the presence and absence of selected organisms (Staphylococcus aureus (ATCC No. 6538), Escherichia coli (ATCC No. 8739), Candida albicans (ATCC No. 10231) and Aspergillus niger (ATCC No. 16404)) according to USP 23 Antimicrobial Effectiveness Test (AET) procedures. According to results of these studies it has  
10 been discovered that suitable preservatives for oligonucleotide formulations include a combination of methylparaben, propylparaben and phenoxyethanol. The total amount of the preservative combination will depend on the dosage form used but will in general be from about 0.1% to 20% by weight. In topical emulsion compositions of the invention, the preservative combination will be present in an amount from about 0.1% to 10%, from about 0.5% to 8%, or  
15 from about 1% to 5%. In one embodiment, methylparaben and propylparaben will each be present in an amount from about 0.1% to 1% and phenoxyethanol in an amount from about 1 to 5%. In other embodiments, methylparaben, propylparaben and phenoxyethanol will be present in a ratio of about 1:1:5 respectively.

The application of emulsion formulations via dermatological, oral and parenteral routes  
20 and methods for their manufacture have been reviewed in the literature (Idson, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of reasons of ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 245;  
25 Idson, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system  
30 of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a

fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: *Controlled Release of Drugs: Polymers and Aggregate*  
5 *Systems*, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant  
10 molecules (Schott, in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker  
15 (Eds.), 1988, volume 1, p. 245; Block, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to,  
20 ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a  
25 short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not  
30 limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty

alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have  
5 been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides et al., Pharmaceutical Research, 1994, 11, 1385; Ritschel, Meth. Find. Exp. Clin. Pharmacol., 1993, 13, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral  
10 administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides et al., Pharmaceutical Research, 1994, 11, 1385; Ho et al., J. Pharm. Sci., 1996, 85, 138). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the  
15 transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

20 Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories - surfactants,  
25 fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 92). Each of these classes has been discussed above.

In another embodiment, emulsion compositions comprise isopropyl myristate (IPM) as an emollient. IPM emulsions of the invention may be in cream form and incorporate IPM in an  
30 amount from about 1% to 50% by weight, about 5% to 20%, or about 10%. In some cream emulsions, glycerol monostearate serves as the oil phase emulsifier while polyoxyl 40 stearate serves as the water phase emulsifier, each present in an amount from about 1% to 30%, or about 5% to 20%. In one embodiment, glycerol monostearate is present in an amount of about 10% by

weight and polyoxyl 40 stearate in an amount of about 15%. Suitable cream emulsions may further comprise viscosity-increasing agents such as hydroxypropyl methylcellulose. In one embodiment, hydroxypropyl methylcellulose is present in an amount from about 0.01% to about 5%, or from 0.1% to 2%, or about 0.5%.

5           There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged  
10 in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall,  
15 are taken up by macrophages in vivo.

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly transformable and able to pass through such fine pores.

20           Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 245). Important considerations in the  
25 preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes. As  
30 the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell, where the active agent may act.

Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes

present several advantages over other formulations. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

- 5 Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

Liposomes fall into two broad classes. Cationic liposomes are positively charged  
10 liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell's cytoplasm (Wang et al., *Biochem. Biophys. Res. Commun.*, 147 (1987) 980-985).

- 15 Liposomes which are pH sensitive or negatively charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the  
20 target cells (Zhou et al., *Journal of Controlled Release*, 19, (1992) 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic  
25 liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

- Several studies have assessed the topical delivery of liposomal drug formulations to the  
30 skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (e.g. as a solution or as an emulsion) were ineffective (Weiner et al., *Journal of Drug Targeting*, 1992, Vol.2 , 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal



formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis et al., Antiviral Research, 18, 1992, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome<sup>®</sup> I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome<sup>®</sup> II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporin-A into different layers of the skin.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes may be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, e.g. they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the 'head') provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in "Pharmaceutical Dosage Forms," Marcel Dekker, Inc., New York, NY, 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol

ethoxylates, propoxylated alcohols, and ethoxylated/ propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in "Pharmaceutical Dosage Forms," Marcel Dekker, Inc., New York, NY, 1988, p. 285).

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging to one of five broad categories, i.e., surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., Crit. Rev. Ther. Drug Carrier Systems, 1991, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid,

with the result that absorption of oligonucleotides through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee et al., Crit. Rev. Ther. Drug Carrier Systems, 1991, p.92); and perfluorchemical emulsions, such as FC-43

5 Takahashi et al., J. Pharm. Pharmacol., 1988, 40:252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-  
10 dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C1-10 alkyl esters thereof (e.g., methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., Crit. Rev. Ther. Drug Carrier Systems, 1991, p.92; Muranishi, Crit. Rev. Ther. Drug Carrier Systems, 1990, 7:1; El Hariri et al., J. Pharm. Pharmacol., 1992, 44:651).

15 Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th Ed., Hardman et al. Eds., McGraw-Hill, New York, 1996, pages 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring  
20 components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucolic acid (sodium glucolate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid  
25 (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Swinyard, Chapter 39 In: Remington's Pharmaceutical Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages  
30 782-783; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7:1; Yamamoto et al., J. Pharm. Exp. Ther., 1992, 263:25; Yamashita et al., J. Pharm. Sci., 1990, 79:579).

Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of oligonucleotides through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have  
5 the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, J. Chromatogr., 1993, 618, 315). Chelating agents of the invention include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino  
10 acyl derivatives of beta-diketones (enamines)(Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7:1; Buur et al., J. Control Rel., 1990, 14:43).

Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant  
15 activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7:1). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92); and non-steroidal anti-  
20 inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., J. Pharm. Pharmacol., 1987, 39:621).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi et al, U.S. Patent No. 5,705,188), cationic glycerol derivatives,  
25 and polycationic molecules, such as polylysine (Lollo et al., PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

30 Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (i.e., does not possess biological activity per se) but is recognized as a nucleic acid by in vivo processes that reduce the bioavailability of a nucleic acid having

biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, 5 presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'isothiocyano-stilbene-2,2'-disulfonic acid (Miyao et al., Antisense Res. Dev., 1995, 5, 115; Takakura et al., Antisense & Nucl. Acid Drug Dev., 1996, 6, 10 177).

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the 15 desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); 20 lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrants (e.g., starch, sodium starch glycolate, etc.); and wetting agents (e.g., sodium lauryl sulphate, etc.).

Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral 25 administration that do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols; polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

30 Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic

excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc,  
5 silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically active materials such as, for example, antipruritics, astringents, local  
10 anesthetics or anti inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the composition of present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be  
15 sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the  
20 suspension including, for example, hydroxypropyl methylcellulose, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

The administration of therapeutic or pharmaceutical compositions comprising the liposomes of the invention is believed to be within the skill of those in the art. In general, a patient in need of therapy or prophylaxis is administered a composition comprising a liposomally  
25 formulated bioactive agents in accordance with the invention, commonly in a pharmaceutically acceptable carrier, in doses ranging from 0.01 ug to 100 g per kg of body weight depending on the age of the patient and the severity of the disorder or disease state being treated. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution  
30 or prevention of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual antisense compounds, and can

generally be estimated based on EC50s found to be effective in in vitro and in vivo animal models.

In the context of the invention, the term "treatment regimen" is meant to encompass therapeutic, palliative and prophylactic modalities of administration of one or more liposomal compositions of the invention. A particular treatment regimen may last for a period of time which will vary depending upon the nature of the particular disease or disorder, its severity and the overall condition of the patient, and may extend from once daily to once every 20 years. Following treatment, the patient is monitored for changes in his/her condition and for alleviation of the symptoms of the disorder or disease state. The dosage of the liposomal composition may either be increased in the event the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the symptoms of the disorder or disease state is observed, or if the disorder or disease state has been ablated.

An optimal dosing schedule is used to deliver a therapeutically effective amount of the bioactive agent encapsulated within the liposomes of the invention being administered via a particular mode of administration. The term "therapeutically effective amount," for the purposes of the invention, refers to the amount of oligonucleotide-containing pharmaceutical composition which is effective to achieve an intended purpose without undesirable side effects (such as toxicity, irritation or allergic response). Although individual needs may vary, determination of optimal ranges for effective amounts of pharmaceutical compositions is within the skill of the art. Human doses can be extrapolated from animal studies (Katocs et al., Chapter 27 In: Remington's Pharmaceutical Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990). Generally, the dosage required to provide an effective amount of a pharmaceutical composition, which can be adjusted by one skilled in the art, will vary depending on the age, health, physical condition, weight, type and extent of the disease or disorder of the recipient, frequency of treatment, the nature of concurrent therapy (if any) and the nature and scope of the desired effect(s) (Nies et al., Chapter 3 In: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th Ed., Hardman et al., eds., McGraw-Hill, New York, NY, 1996).

Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the bioactive agent is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years. For example, in the case of an individual known or suspected of being prone to an autoimmune or inflammatory condition, prophylactic effects may be achieved by administration of preventative doses, ranging from 0.01 ug to 100 g per kg of

body weight, once or more daily, to once every 20 years. In like fashion, an individual may be made less susceptible to an inflammatory condition that is expected to occur as a result of some medical treatment, e.g., graft versus host disease resulting from the transplantation of cells, tissue or an organ into the individual.

5 Prophylactic modalities for high risk individuals are also encompassed by the invention. As used herein, the term "high risk individual" is meant to refer to an individual for whom it has been determined, via, e.g., individual or family history or genetic testing, that there is a significantly higher than normal probability of being susceptible to the onset or recurrence of a disease or disorder. For example, a subject animal could have a personal and/or family medical  
10 history that includes frequent occurrences of a particular disease or disorder. As another example, a subject animal could have had such a susceptibility determined by genetic screening according to techniques known in the art (see, e.g., U.S. Congress, Office of Technology Assessment, Chapter 5 In: Genetic Monitoring and Screening in the Workplace, OTA-BA-455, U.S. Government Printing Office, Washington, D.C., 1990, pages 75-99). As part of a treatment  
15 regimen for a high risk individual, the individual can be prophylactically treated to prevent the onset or recurrence of the disease or disorder. The term "prophylactically effective amount" is meant to refer to an amount of a pharmaceutical composition which produces an effect observed as the prevention of the onset or recurrence of a disease or disorder. Prophylactically effective amounts of a pharmaceutical composition are typically determined by the effect they have  
20 compared to the effect observed when a second pharmaceutical composition lacking the active agent is administered to a similarly situated individual.

From in vivo animal studies wherein oligonucleotides have been administered topically or intradermally it has been shown that oligonucleotides become widely distributed from the site of administration. For example oligonucleotide ISIS-2302 was topically applied on the back of  
25 mini pigs and rats. Samples of dermal and epidermal tissue analyzed by capillary gel electrophoresis and immunohistochemical staining detected significant levels of the oligonucleotide not only at the administration site (back) but also on stomach, neck and hind leg. Accordingly there is provided a method for delivering an oligonucleotide to a first dermal or epidermal tissue site in an animal comprising applying said oligonucleotide to a second dermal  
30 or epidermal tissue site in said animal wherein said first site is removed from said second site. In some embodiments, the oligonucleotide is administered topically in a pharmaceutical composition of the invention, in particular in an emulsion as described herein. The method is particularly useful for ensuring delivery of oligonucleotide evenly to dermal or epidermal tissue



and/or over a great area or to sites that would otherwise be difficult to apply or would be sensitive to direct administration.

#### *Sources of Compounds*

In general, the compounds used in the studies described herein are available from a variety of commercial sources, or can be synthesized from available reagents by those skilled in the art using methods known in the art. For sake of convenience, some specific commercial suppliers of the more significant compounds used in, or identified by, the studies described herein are provided in the following list.

Chol (cholesterol) is purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) or from Sigma Chemical Corp. (St. Louis, MO).

1-Dodecyl-2-pyrrolidinone is purchased from Aldrich Chemical Co. (Milwaukee, WI).

DOPE (dioleoylphosphatidylethanolamine) is purchased from Avanti.

DMPC (dimyristoylphosphatidylcholine) is purchased from Avanti or Sigma.

DPPC (dipalmitoylphosphatidylcholine) is purchased from Sigma, Avanti or Genzyme Corp. (Cambridge, MA).

DMPG (dimyristoylphosphatidylglycol) is purchased from Avanti or Sigma.

DMSO (dimethyl sulfoxide) is purchased from Sigma or Aldrich.

IPM (isopropyl myristate, a.k.a. myristic acid isopropyl ester) is purchased from Sigma or Aldrich.

Menthone is purchased from Aldrich.

1-Methyl-2-pyrrolidinone is purchased from Sigma.

Oleic acid is purchased from Sigma.

PG (propylene glycol, a.k.a. 1,2-propanediol) is purchased from Sigma or Aldrich.

Tween 40 [polyoxyethylene (2)sorbitan monopalmitate] is purchased from Sigma or Aldrich.

Azone (dodecyl azone, a.k.a. laurocapram) is purchased from Shanghai Daniel Chem Technologies Co., Ltd., Shanghai, People's Republic of China.

Limonene (d-limonene) is purchased from Sigma.

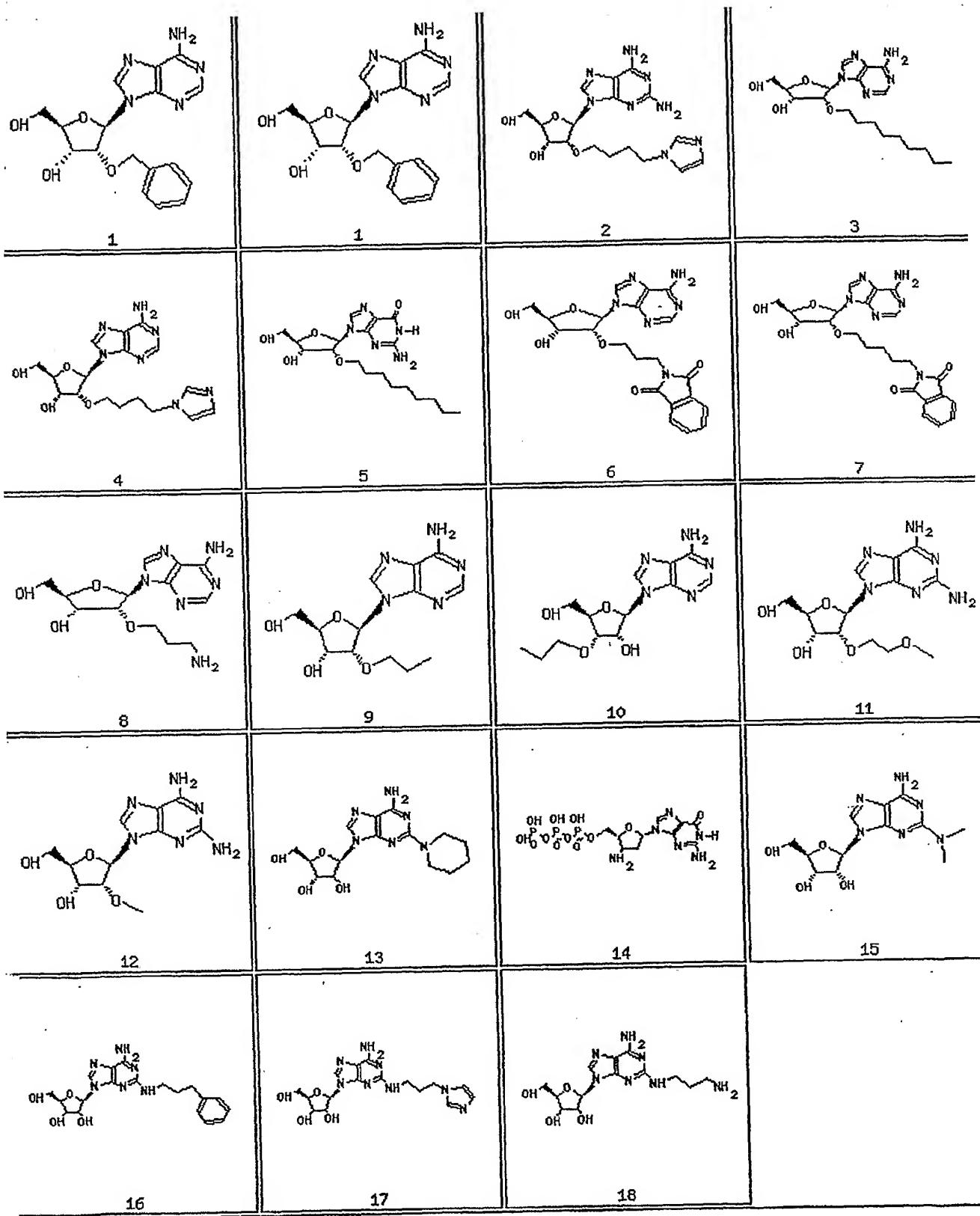
MIGLYOLTM 818 is purchased from Hhls AG, Marl, Germany.

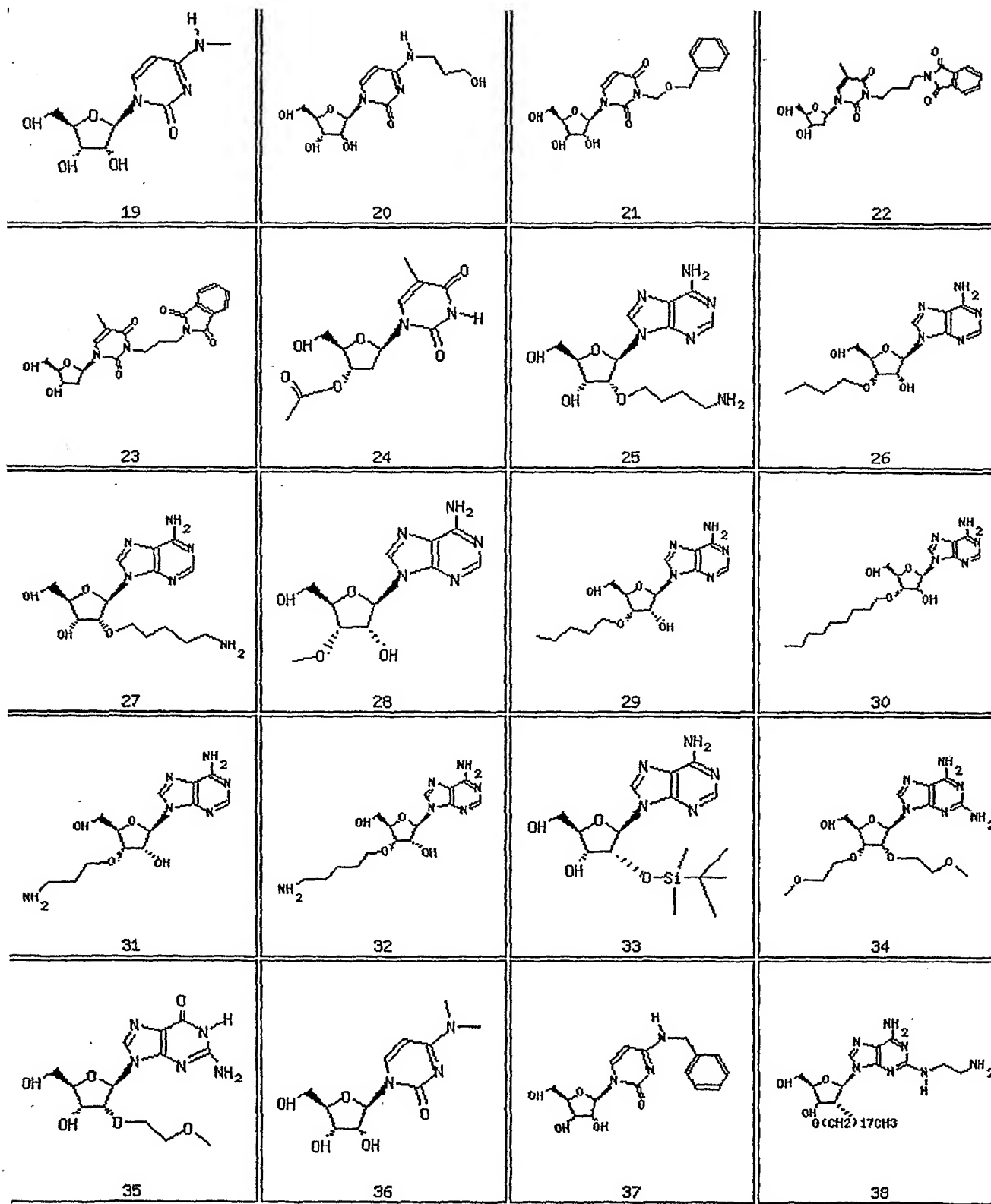
#### **Example 11: Representative Antiviral Nucleosides and Nucleoside Mimetics**

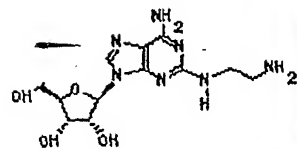
Suitable compounds are shown in Table 1. As stated herein, the term "antiviral nucleoside and/or nucleoside mimetic" refers to any one or more of the nucleosides or nucleoside mimetics shown in Table 1.

Various modifications of the invention, in addition to those described herein, will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. Each reference (i.e., U.S. Patent, U.S. Patent application, PCT International Publication, journal article, and the like) cited in or  
5 referred to in the present application is incorporated herein by reference in its entirety. U.S. provisional application Serial No. 60/472,774 filed May 21, 2003 is incorporated herein by reference in its entirety.

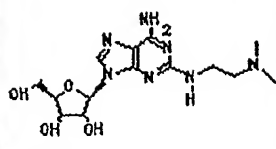
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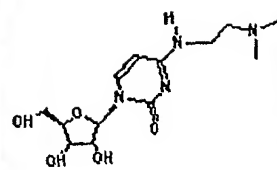




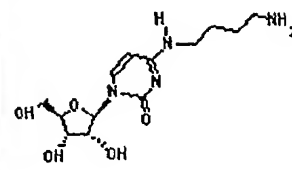
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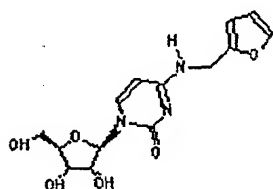
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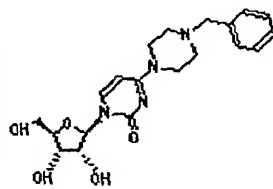
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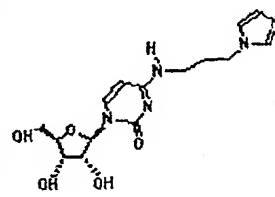
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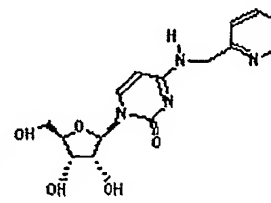
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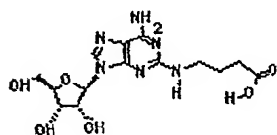
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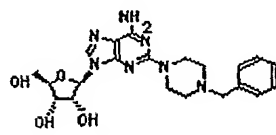
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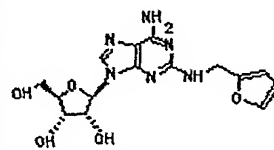
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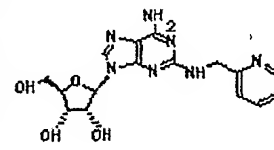
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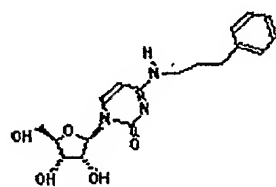
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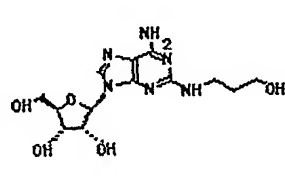
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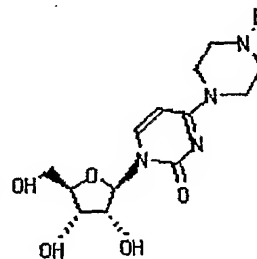
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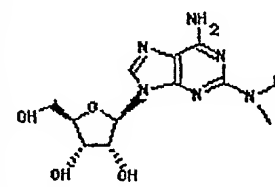
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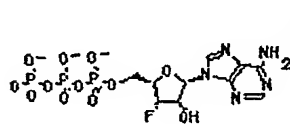
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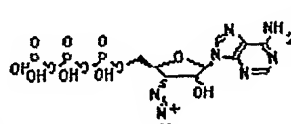
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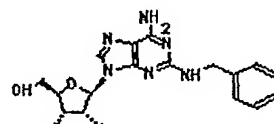
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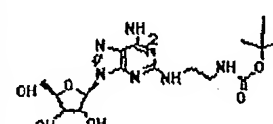
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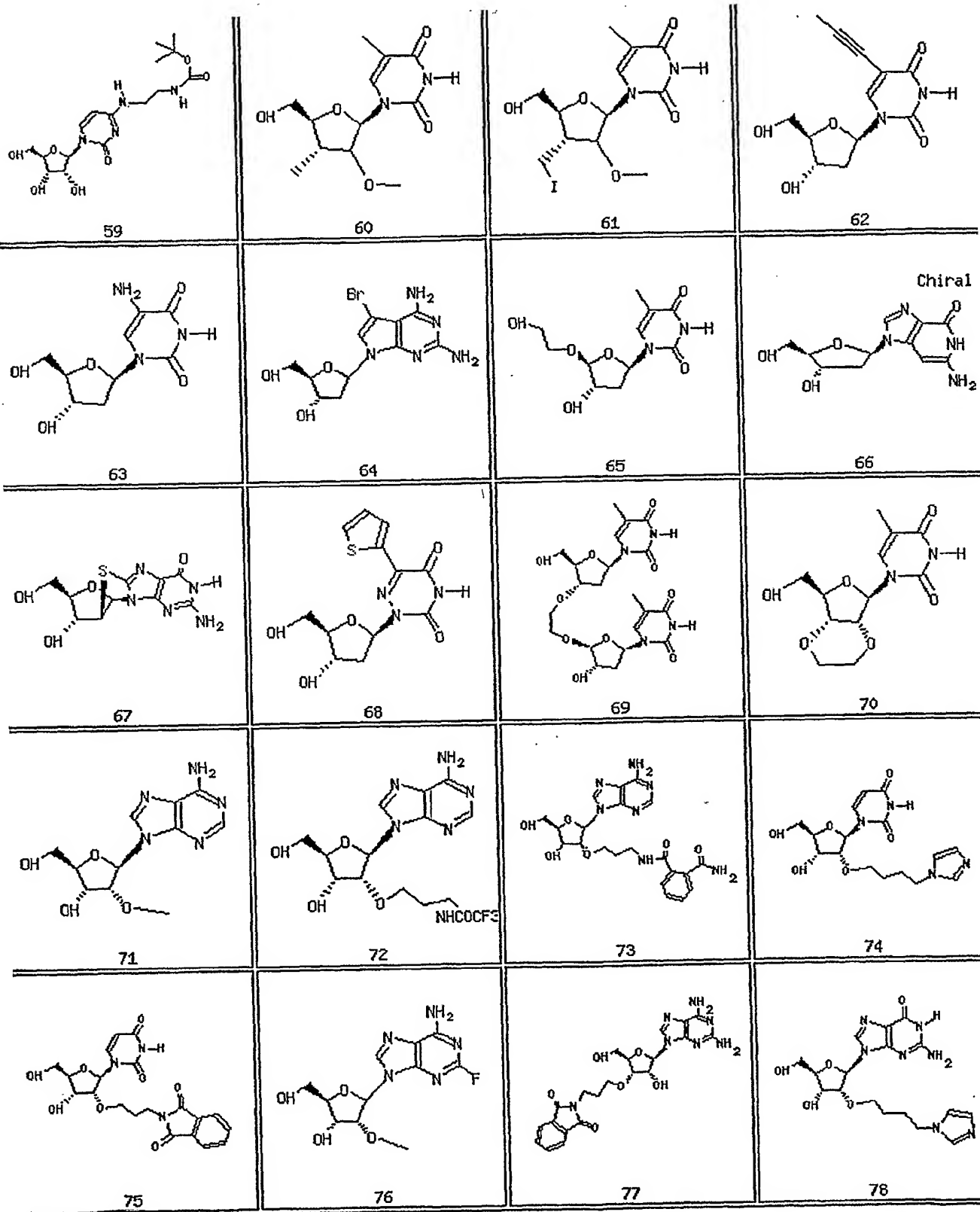
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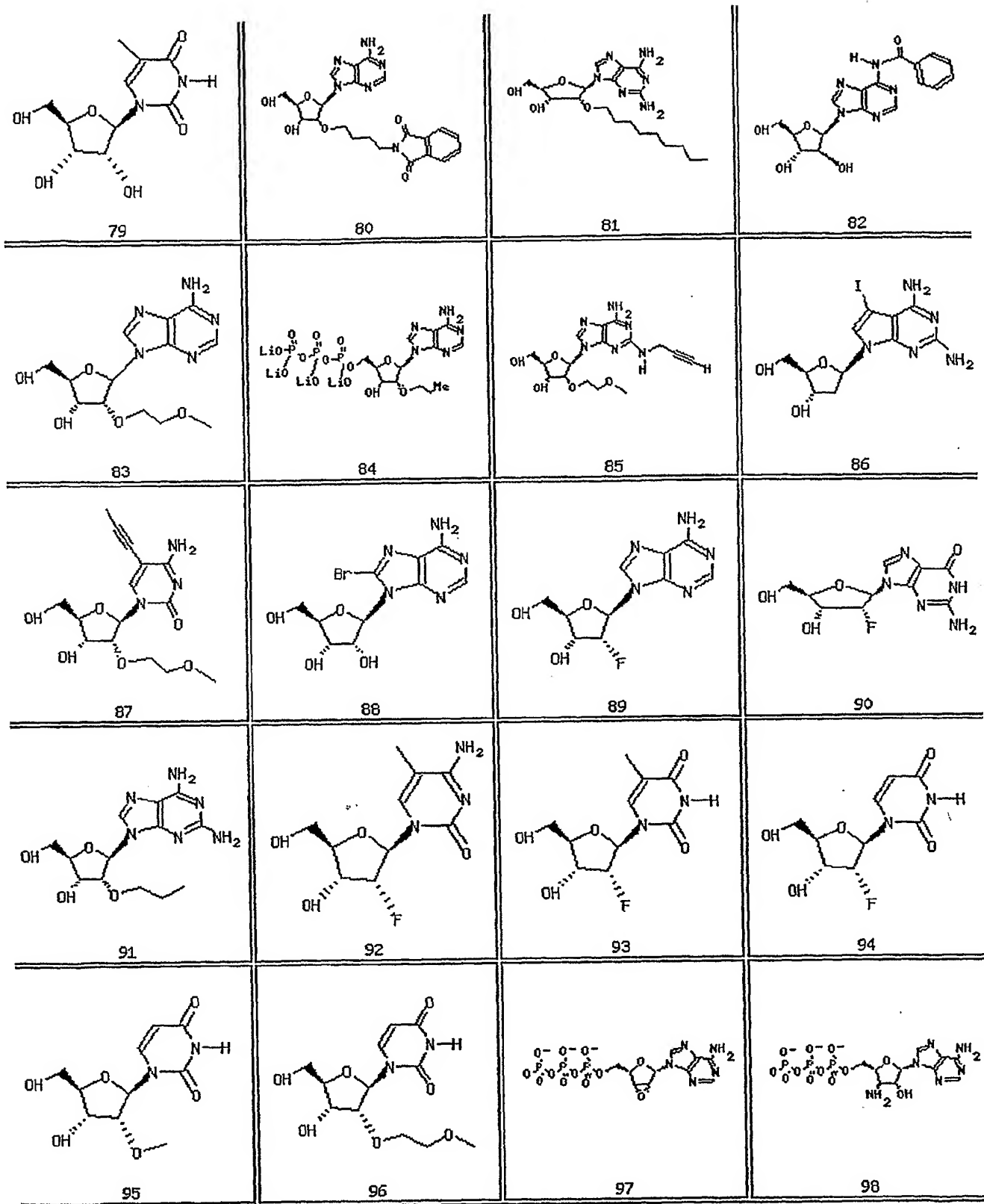


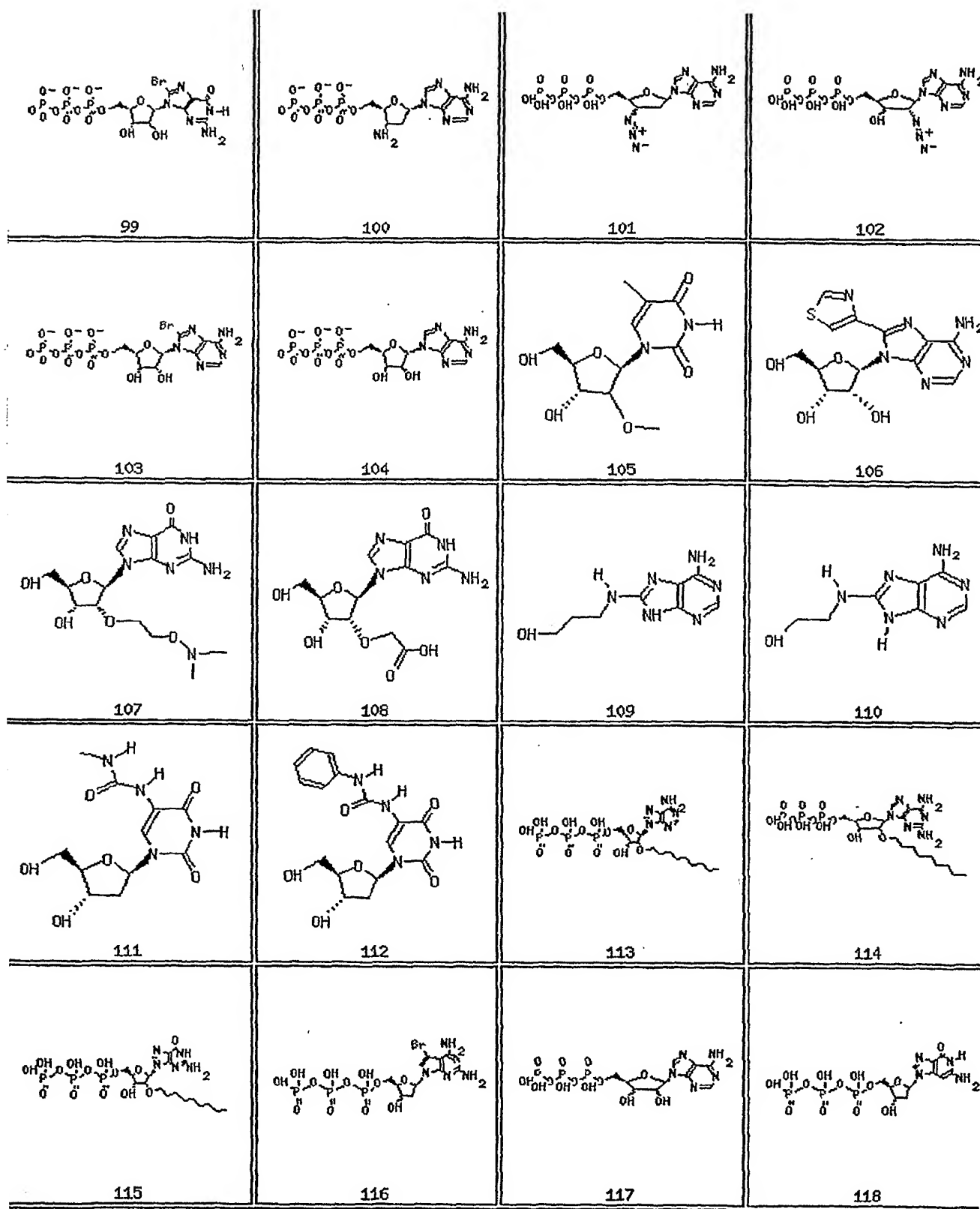
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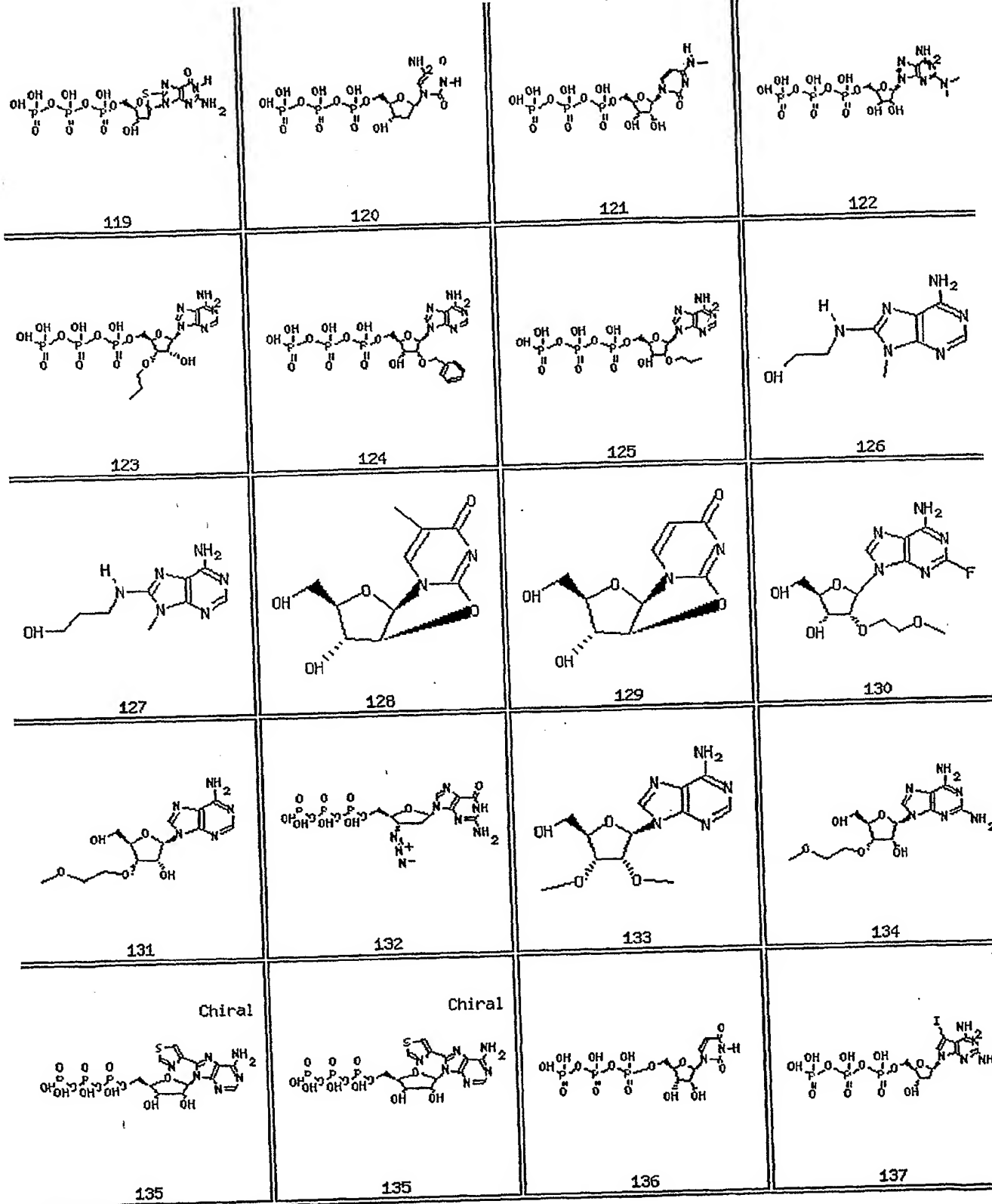
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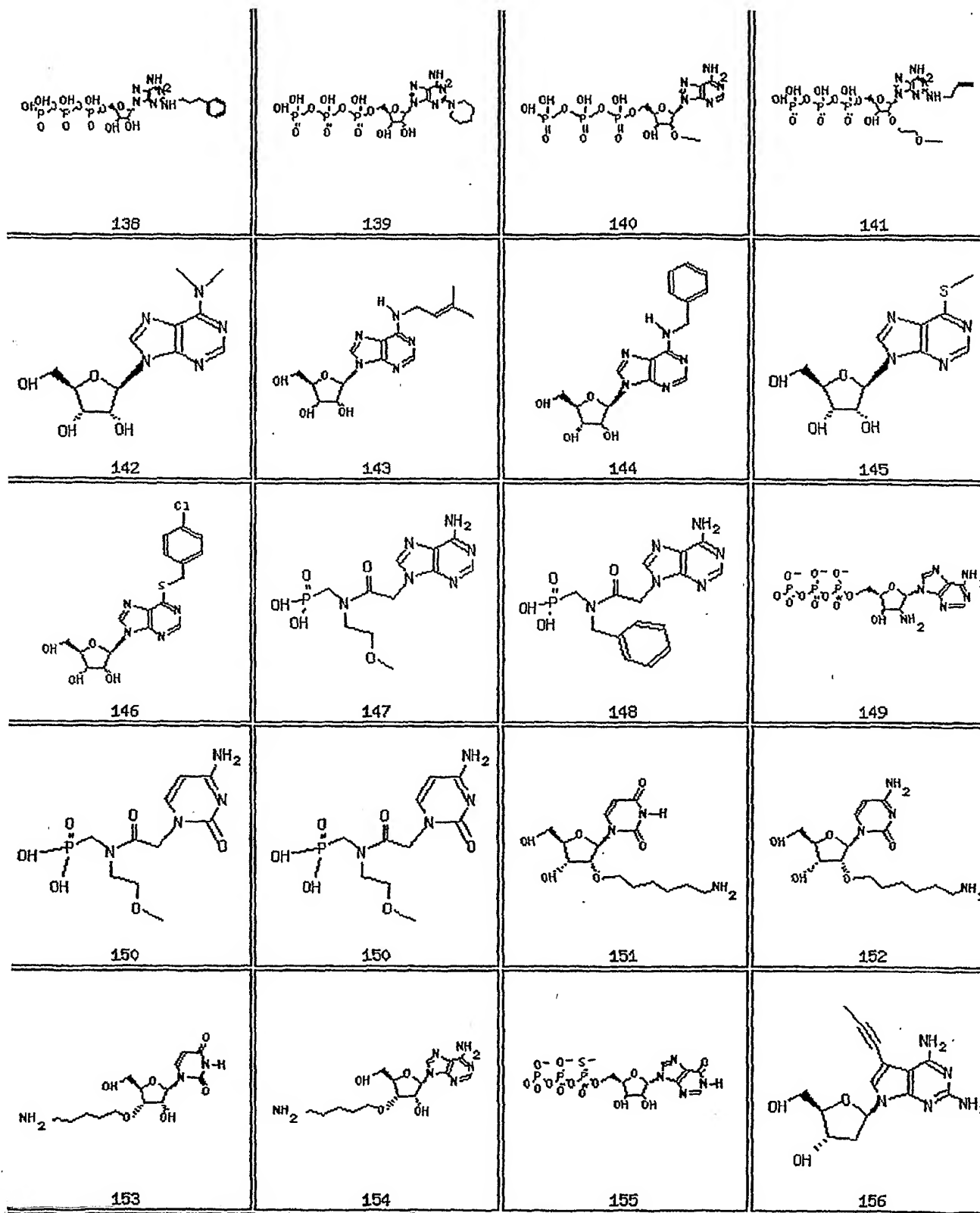


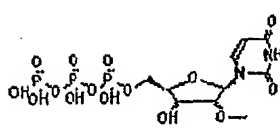
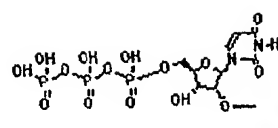
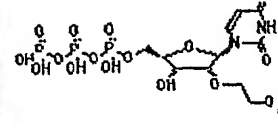
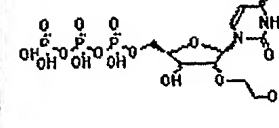
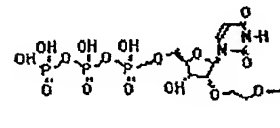
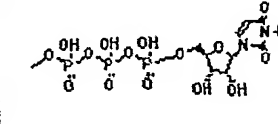
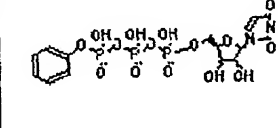
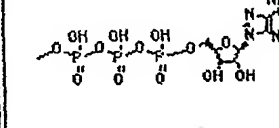
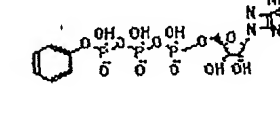
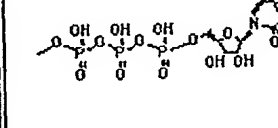
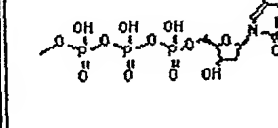
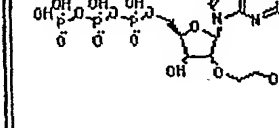
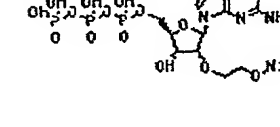
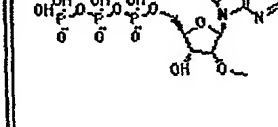
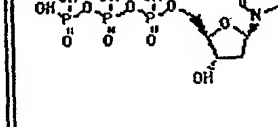
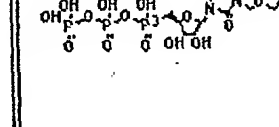
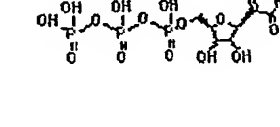
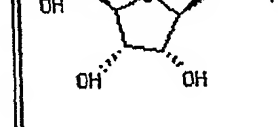
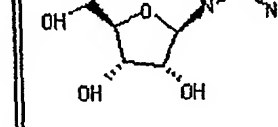
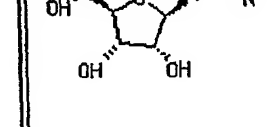


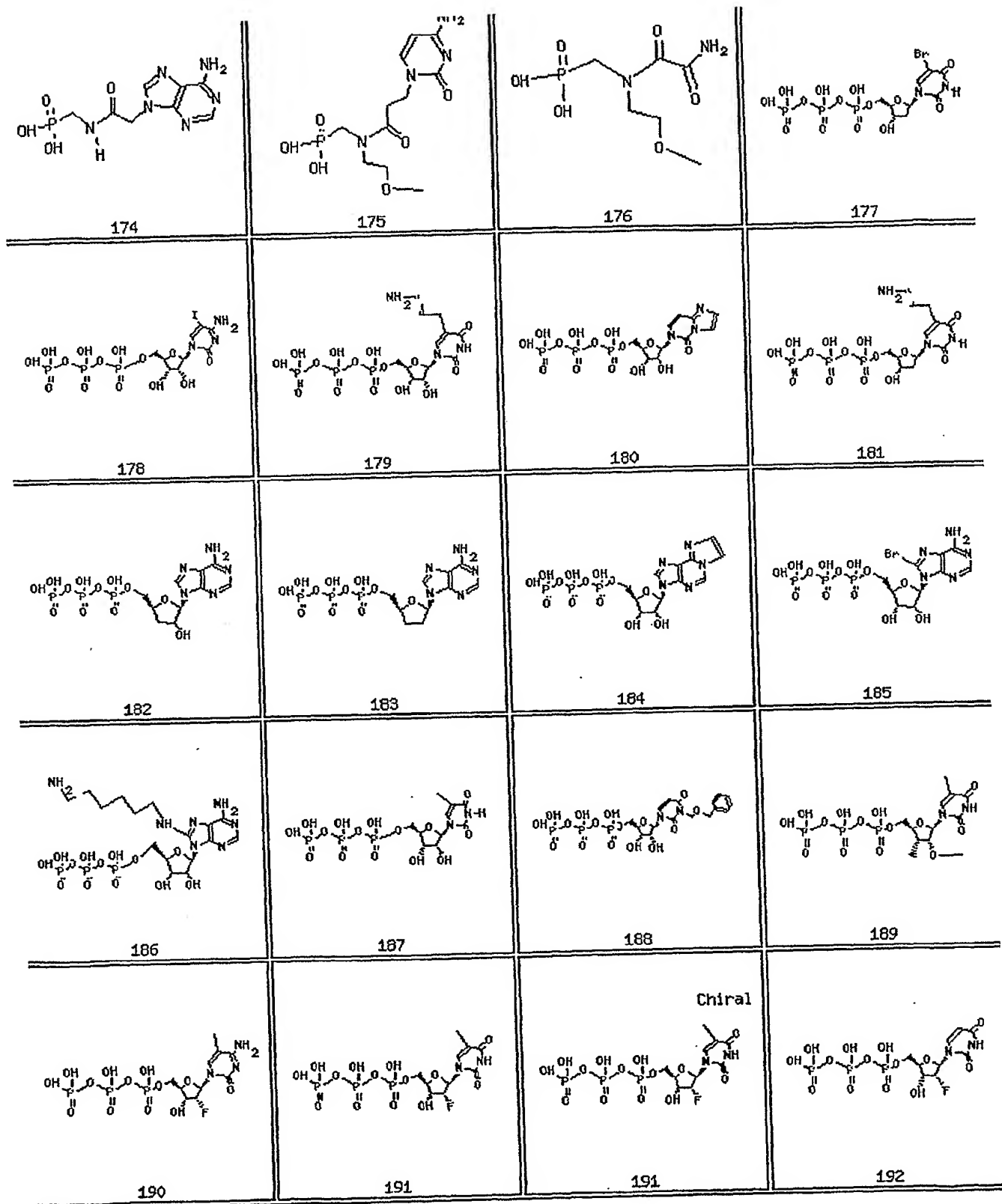


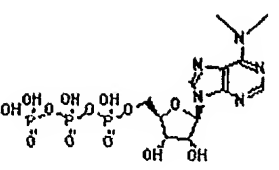
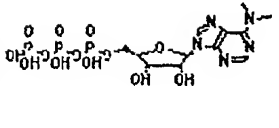
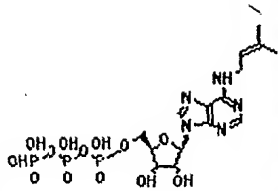
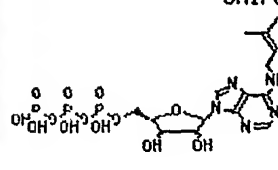
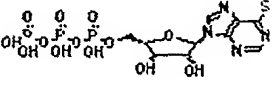
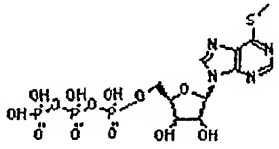
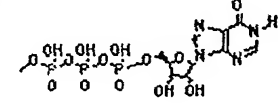
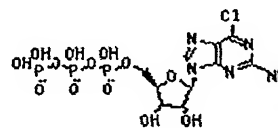
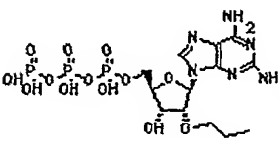
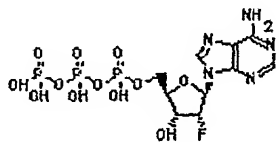
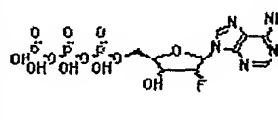
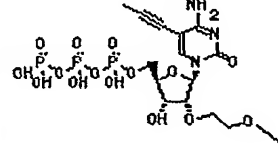
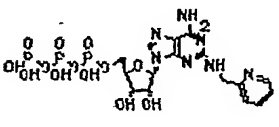
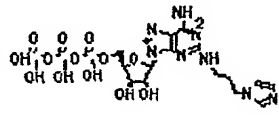
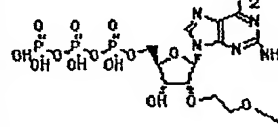
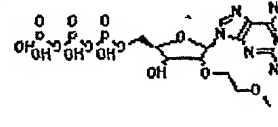
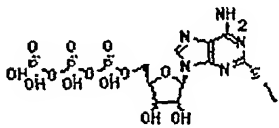
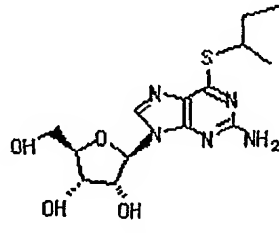
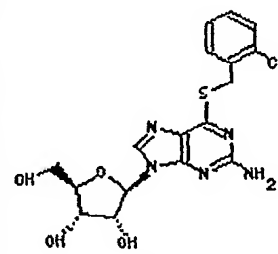
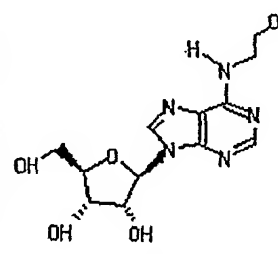


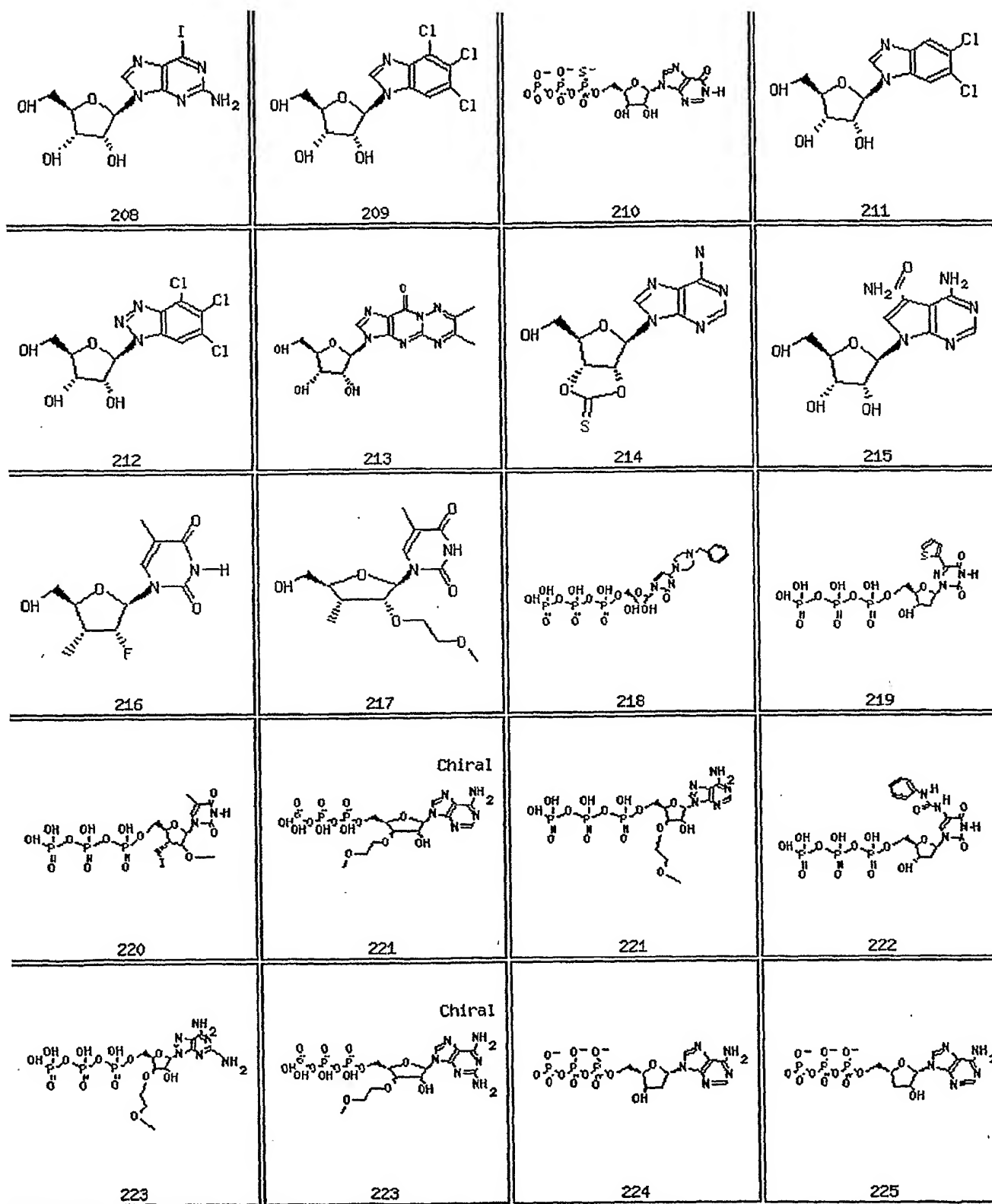


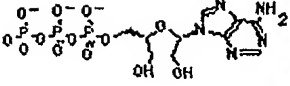
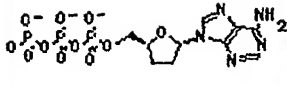
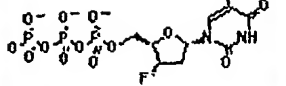
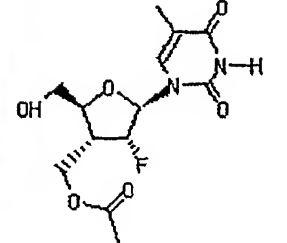
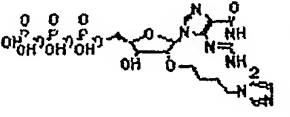
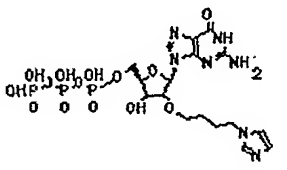
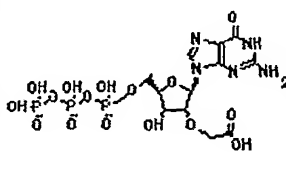
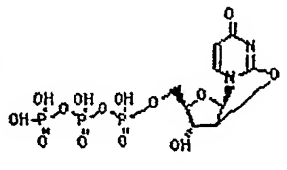
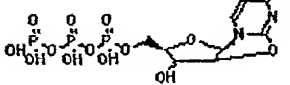
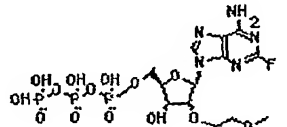
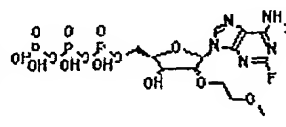
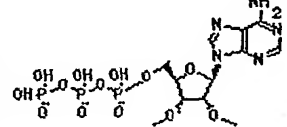
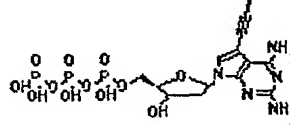
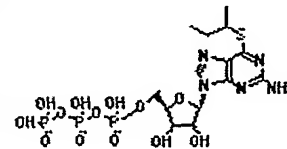
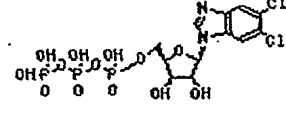
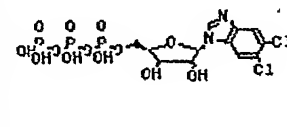
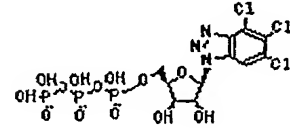
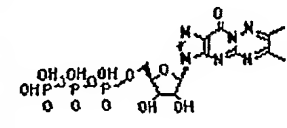
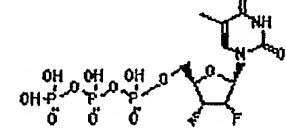
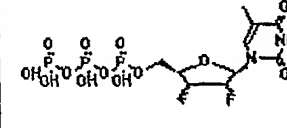


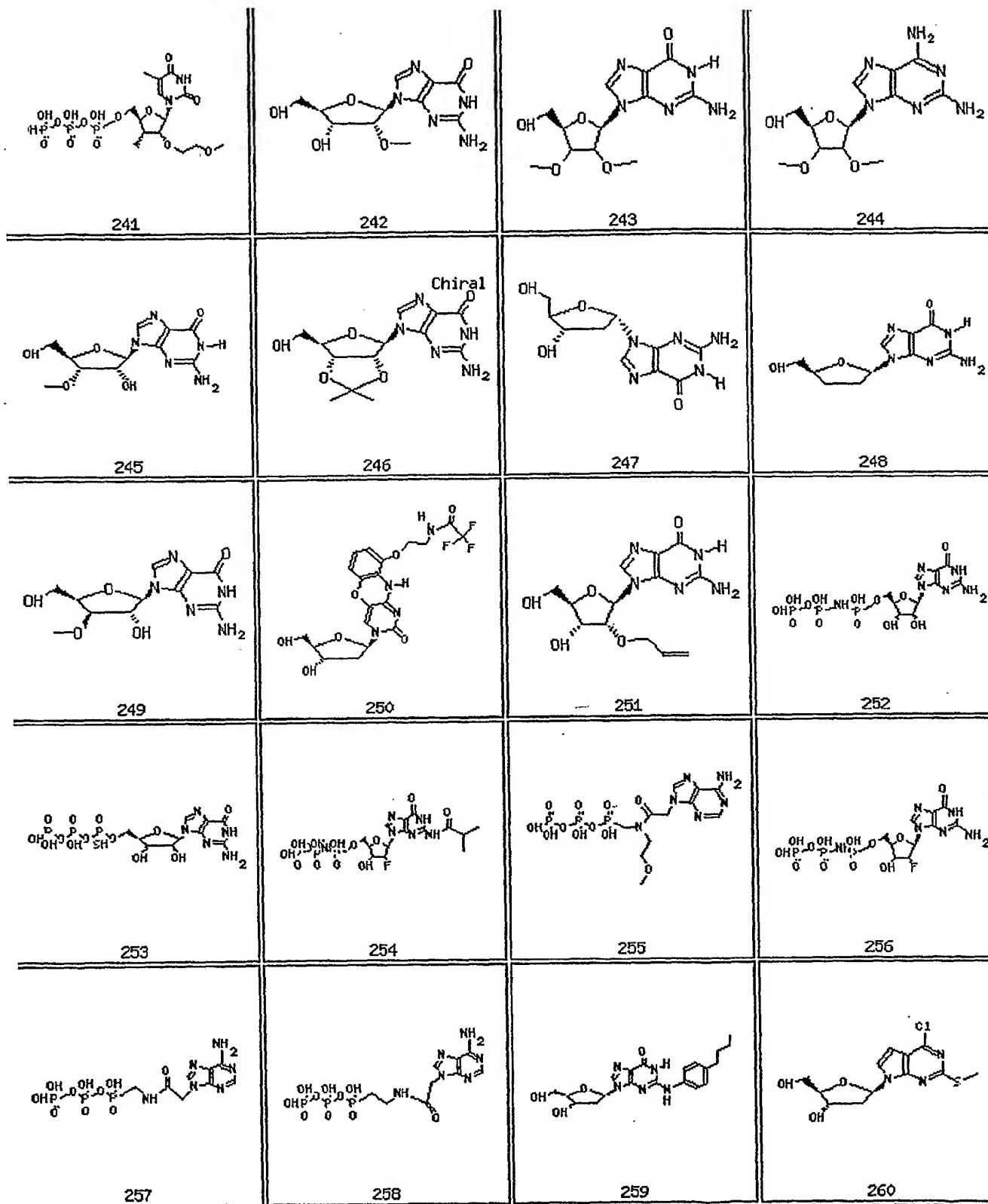
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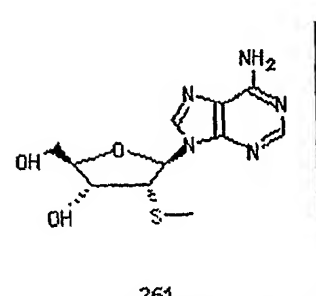
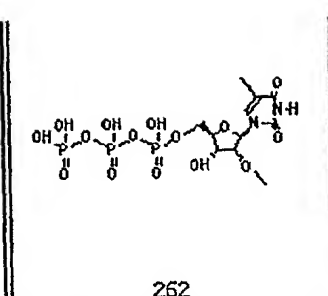
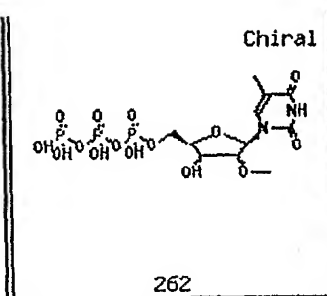
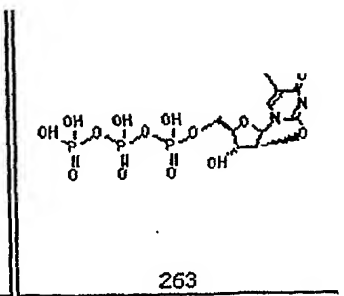
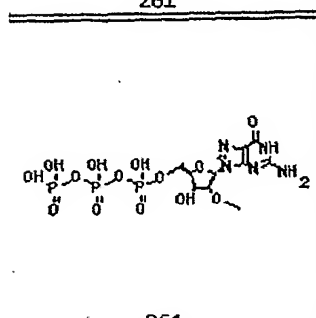
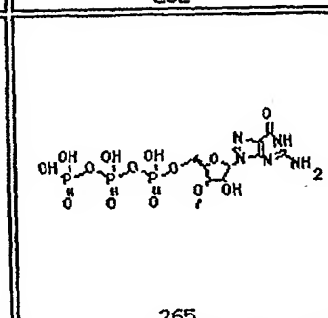
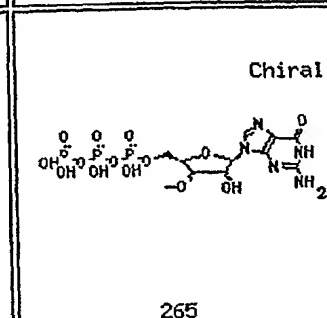
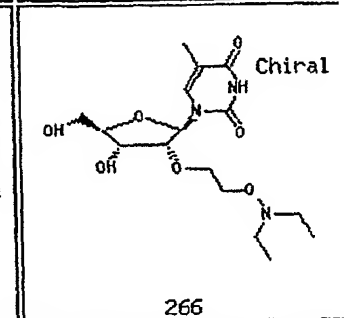
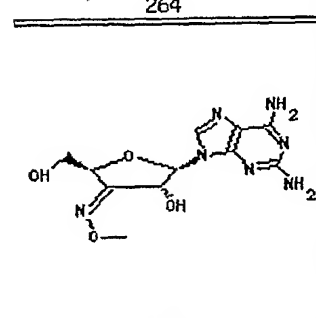
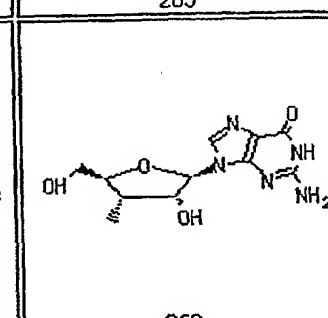
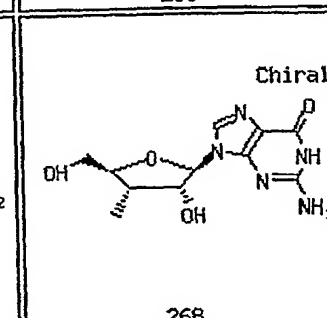
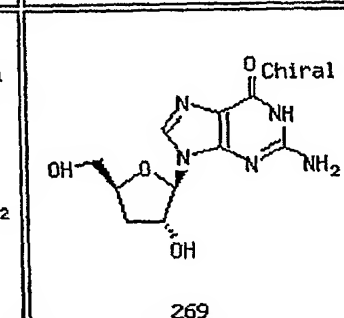
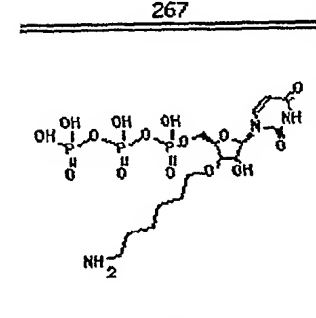
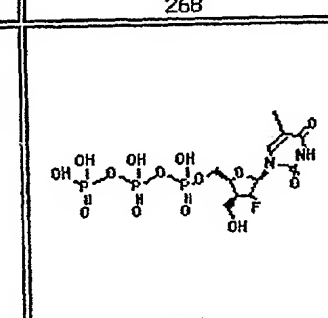
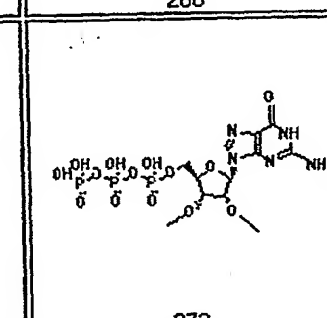
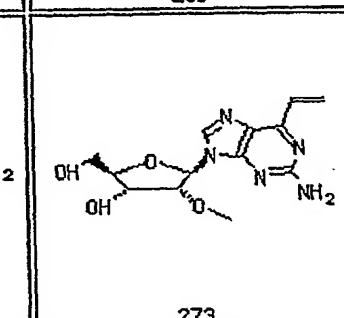
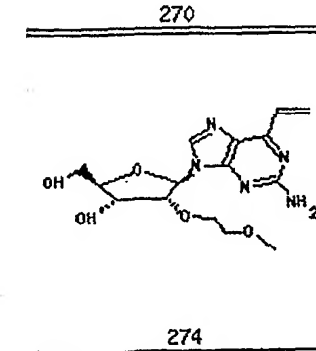
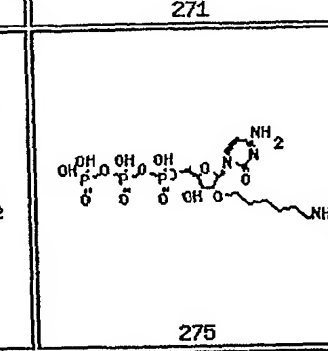
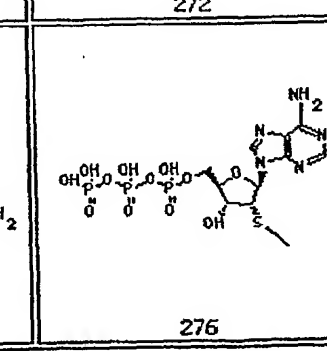
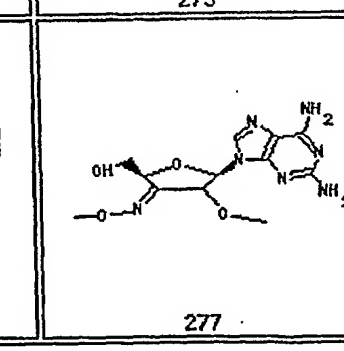
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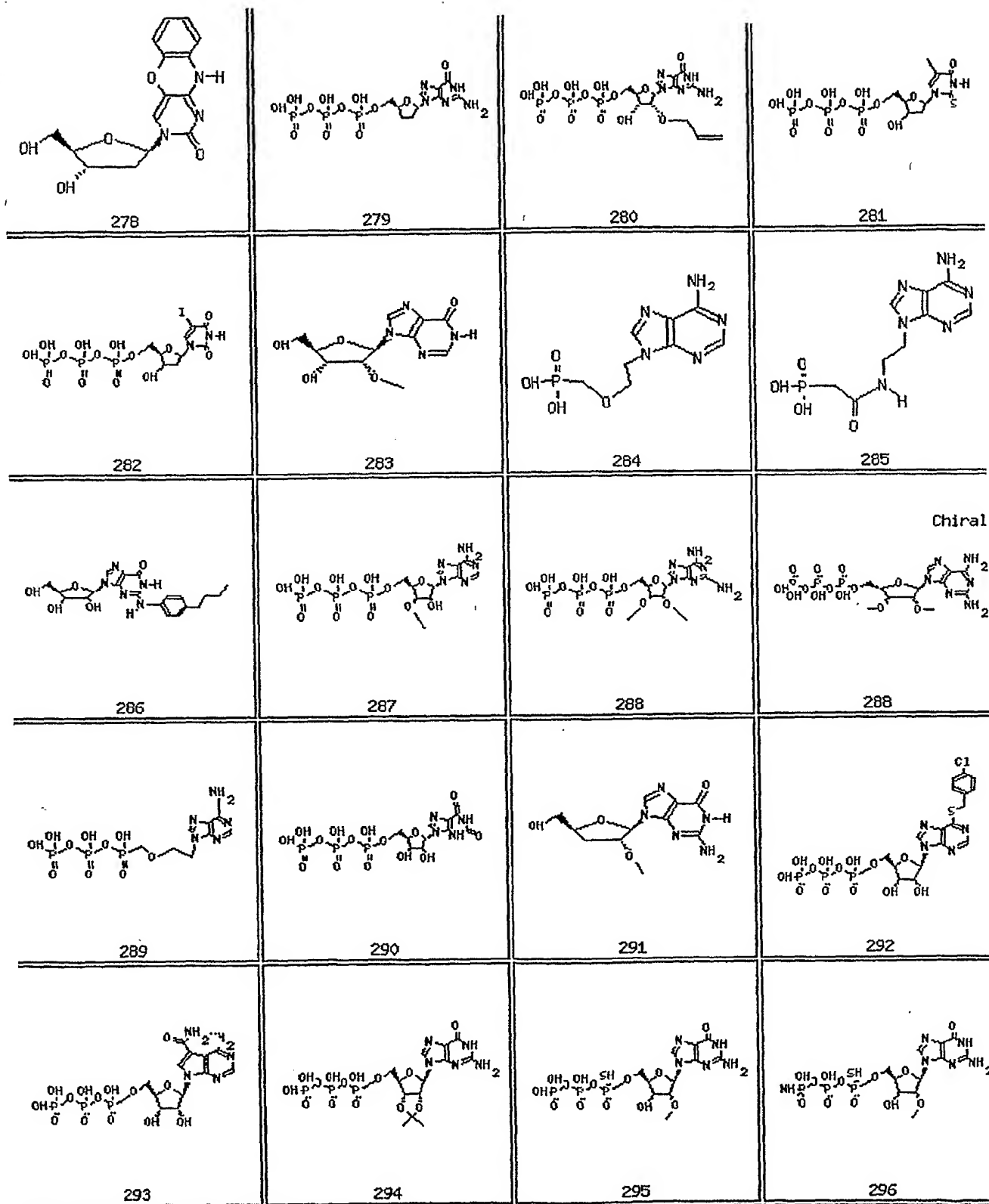


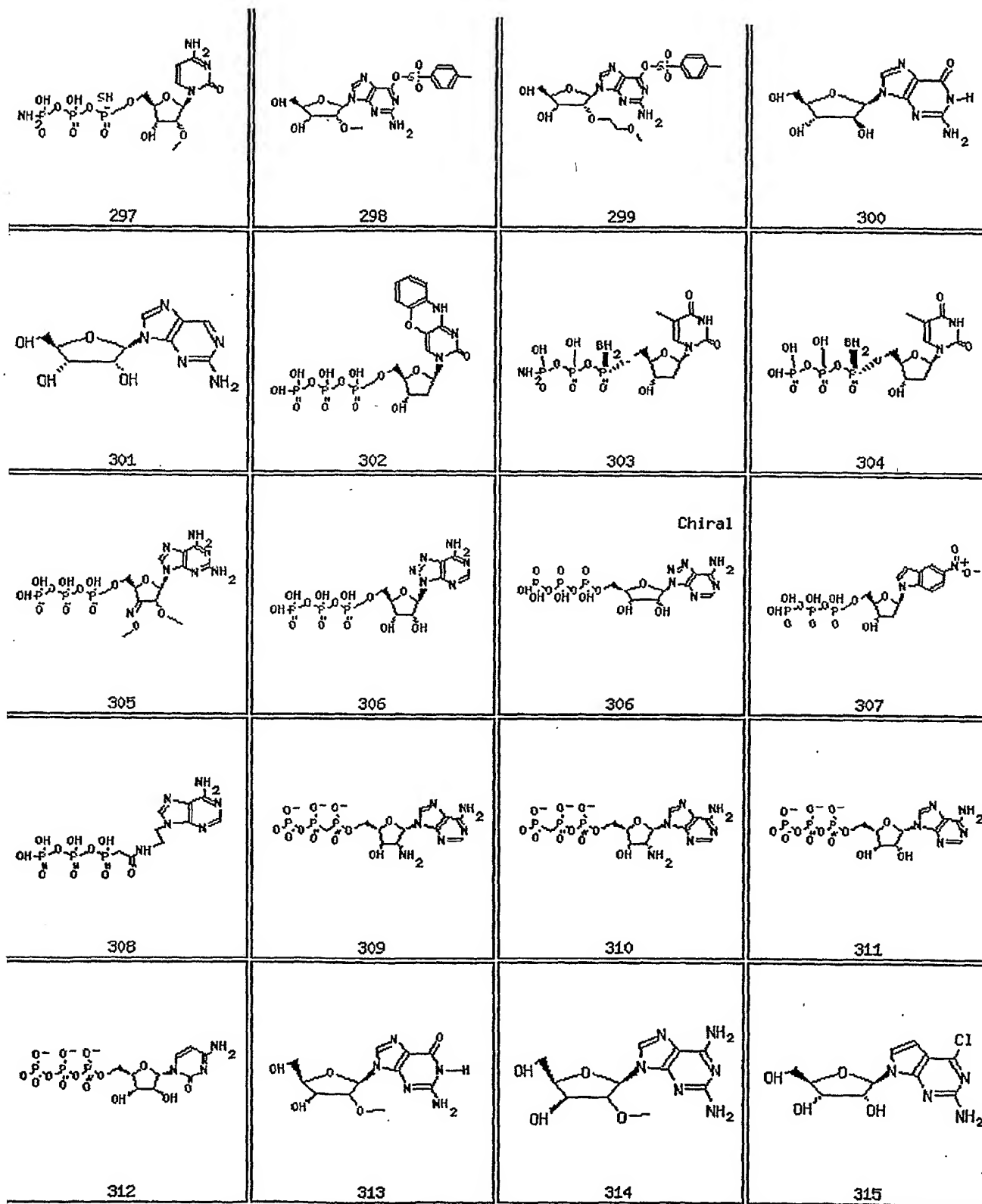
			
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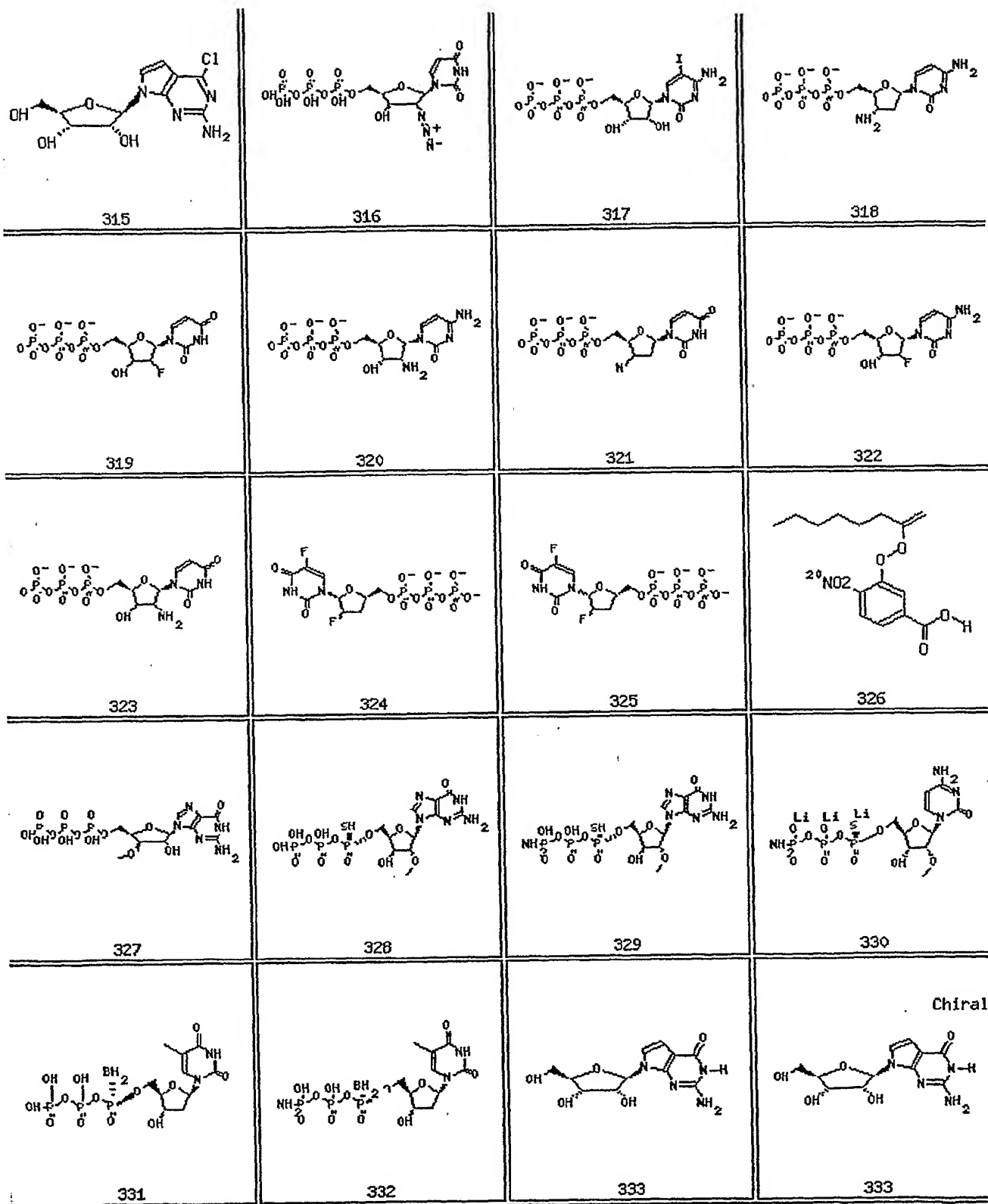


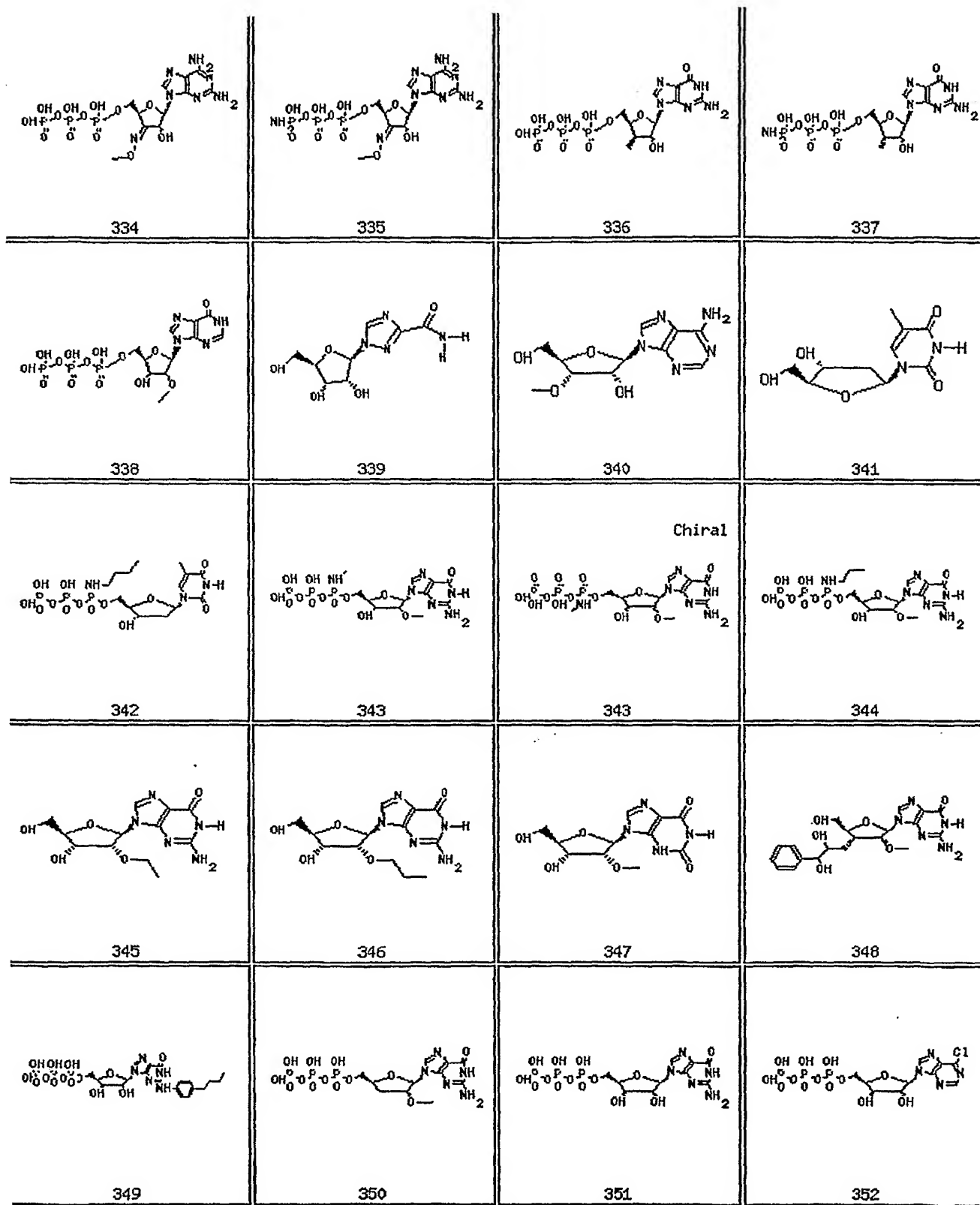


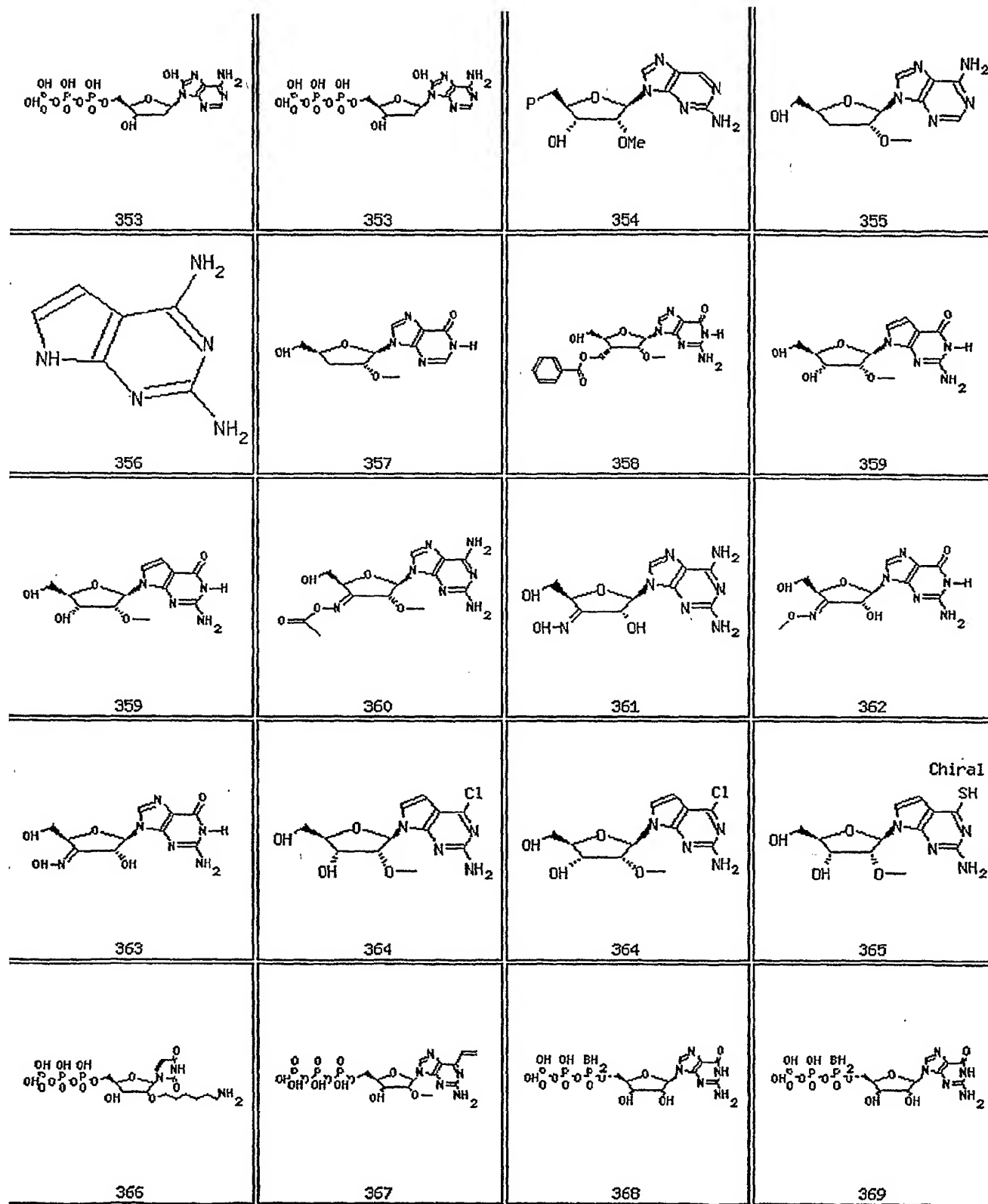
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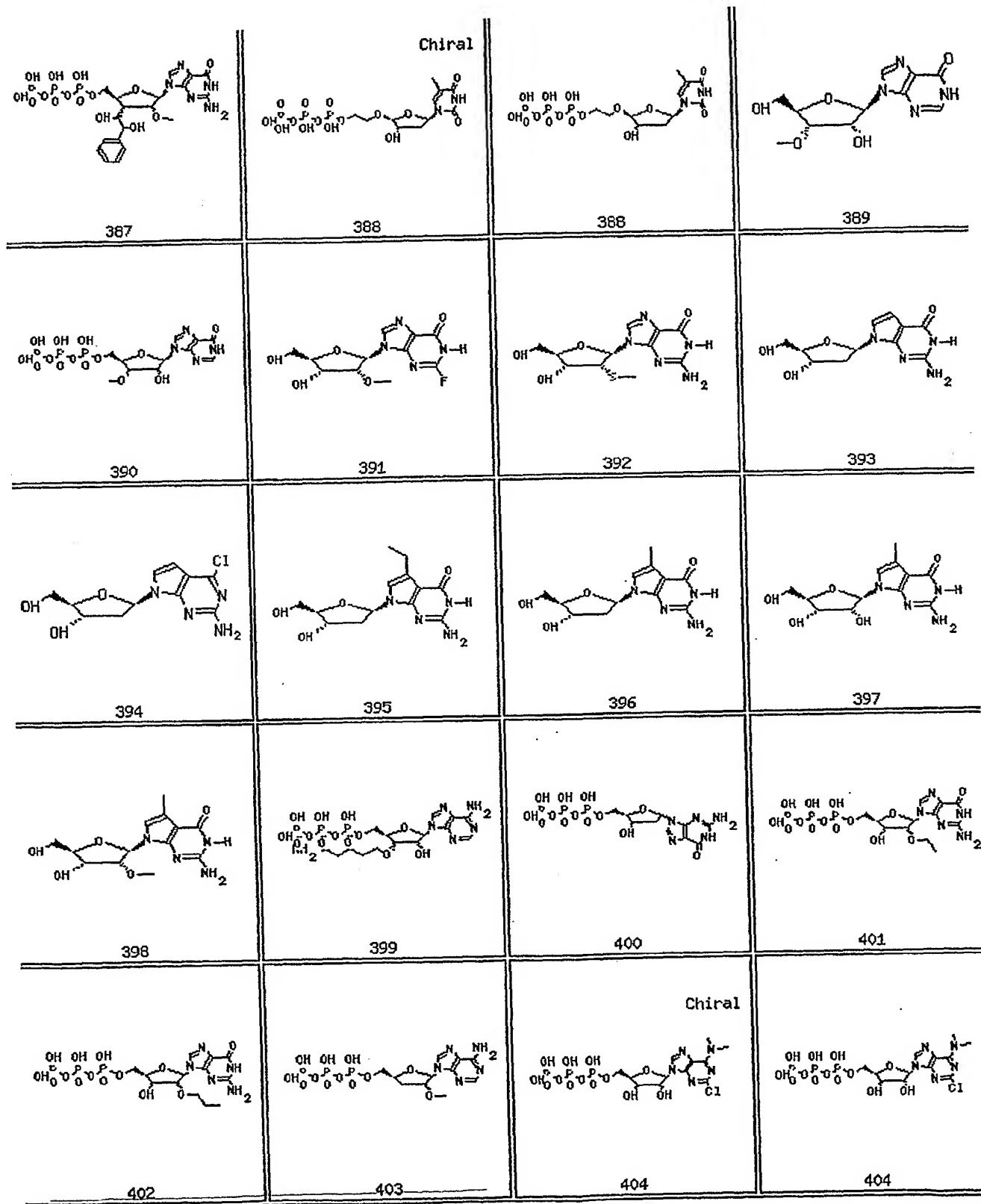




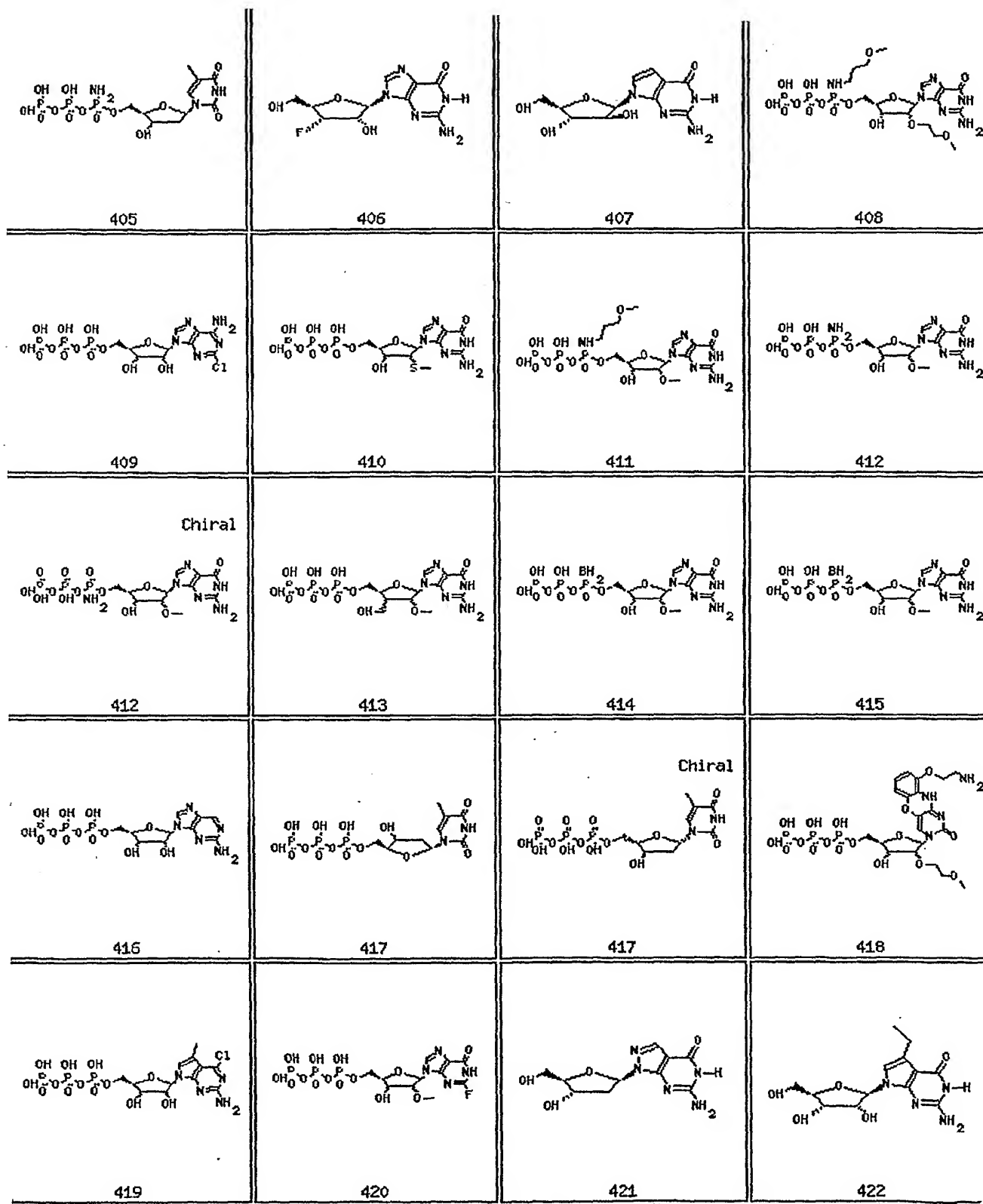


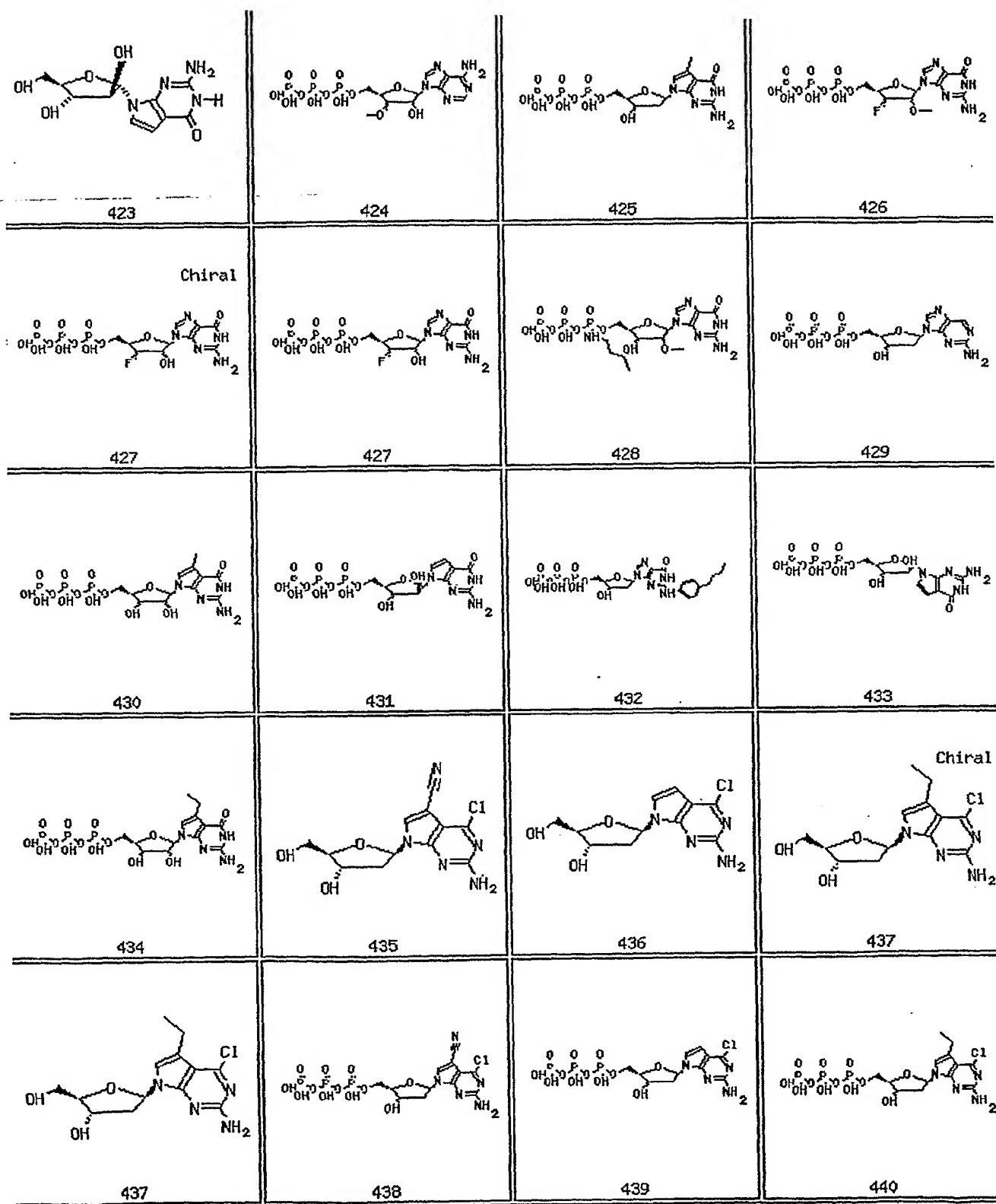


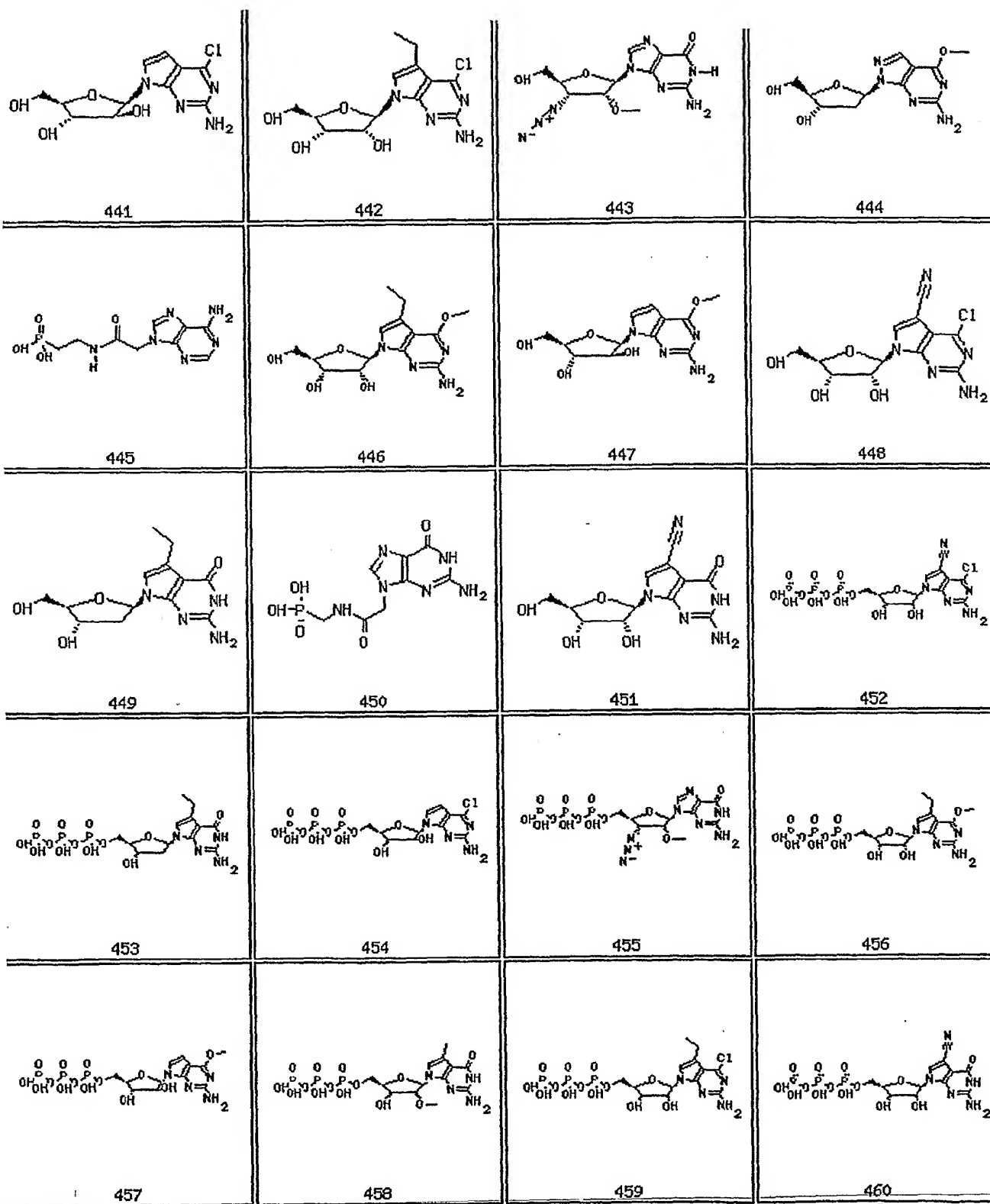
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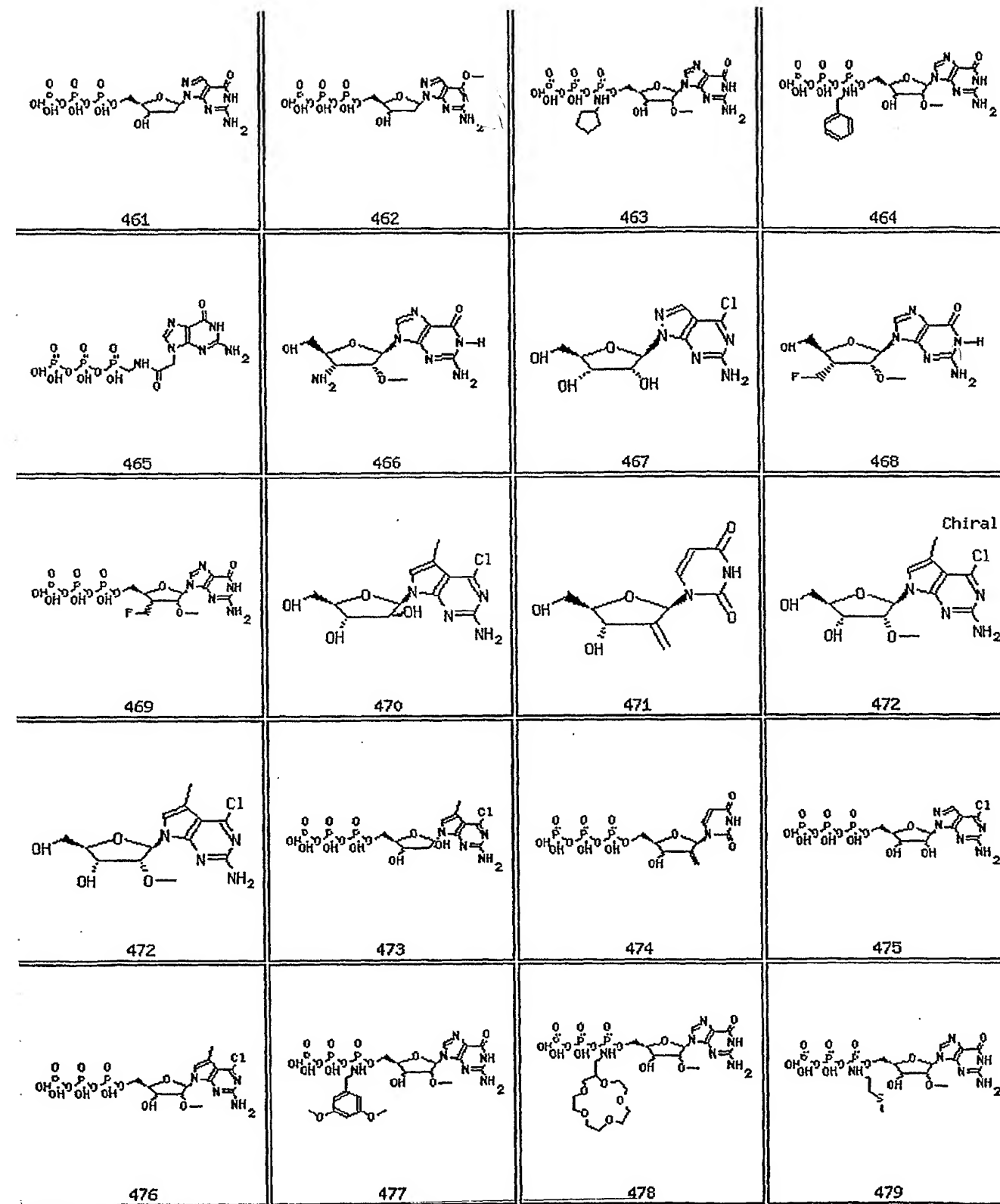


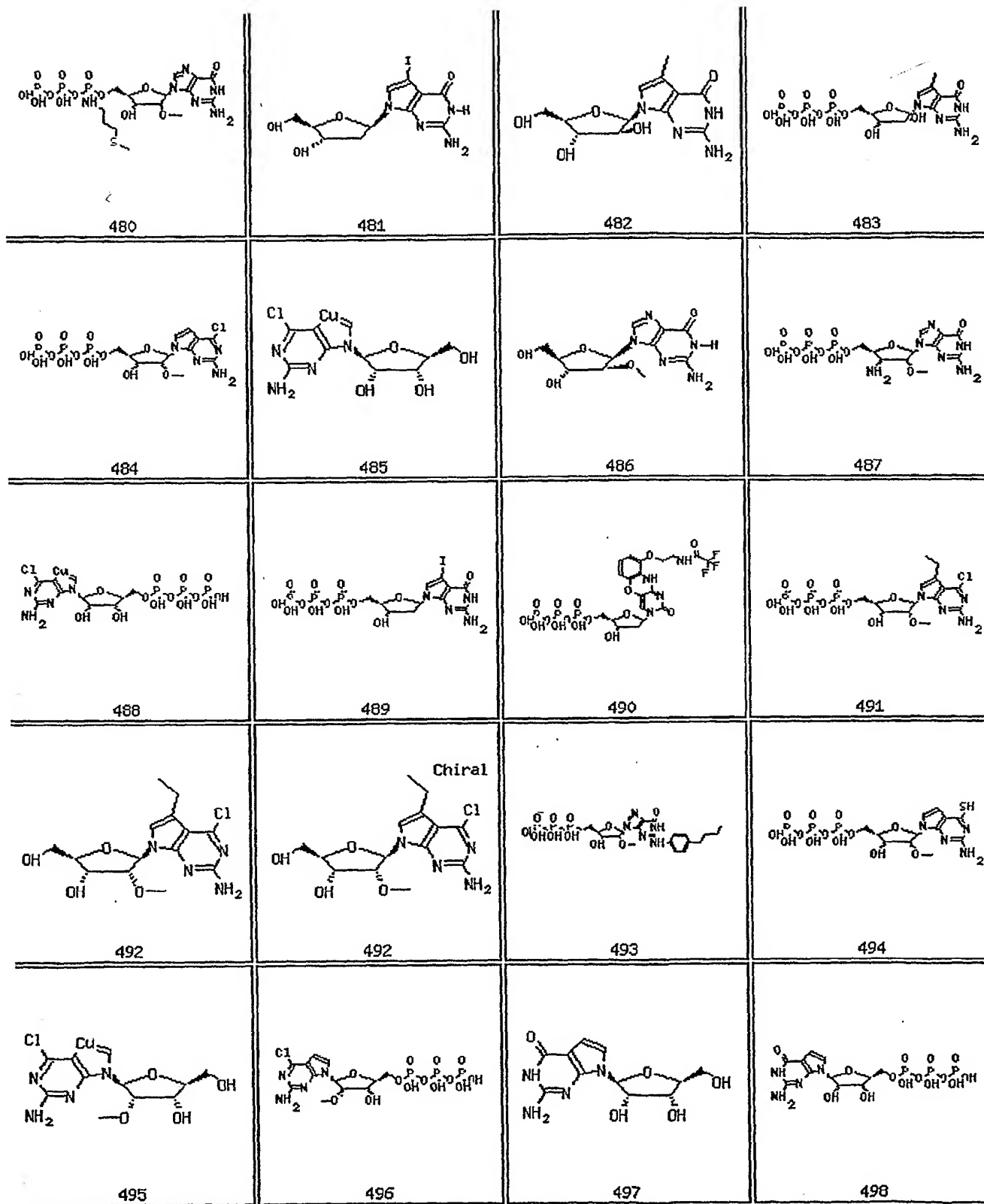


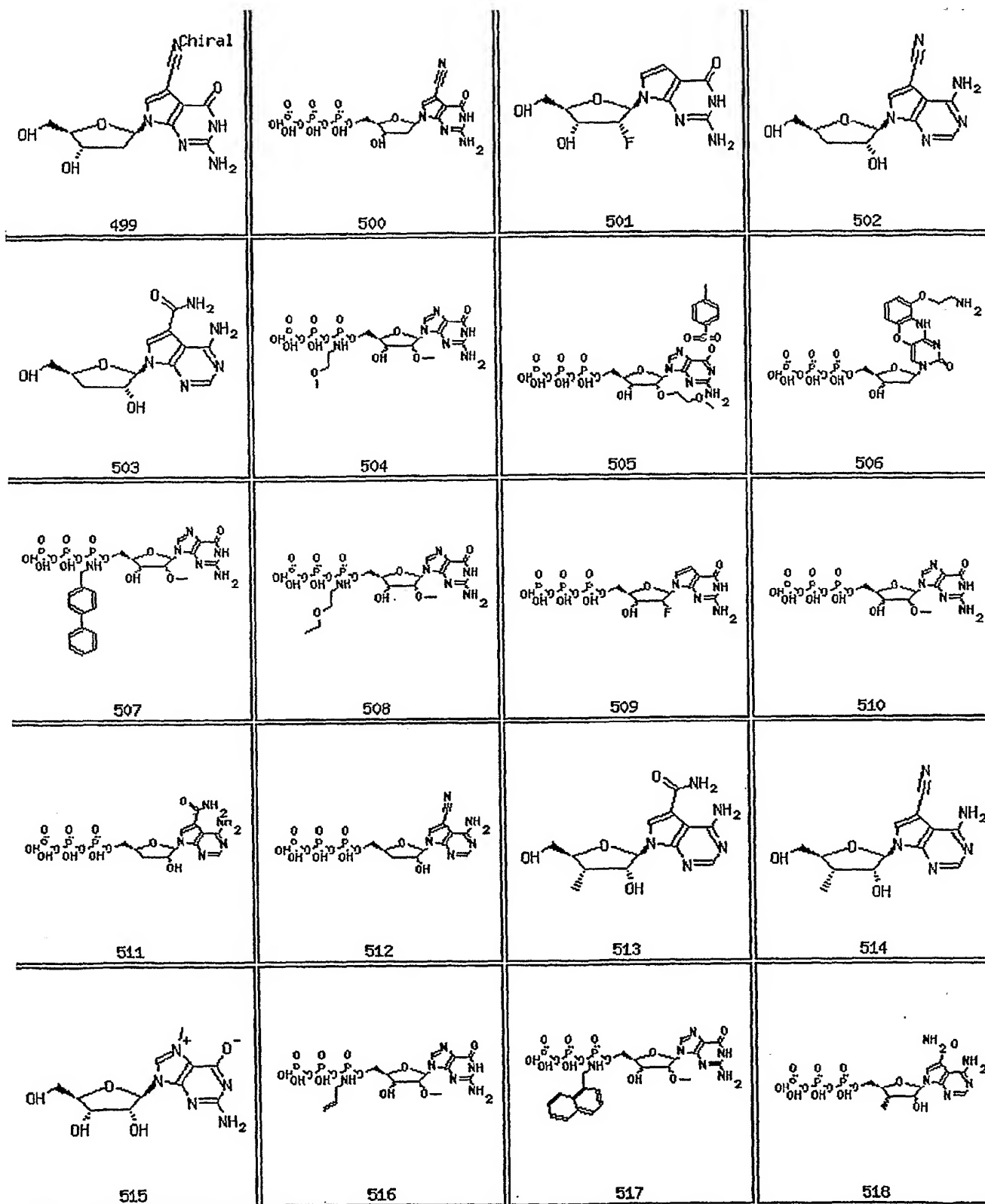


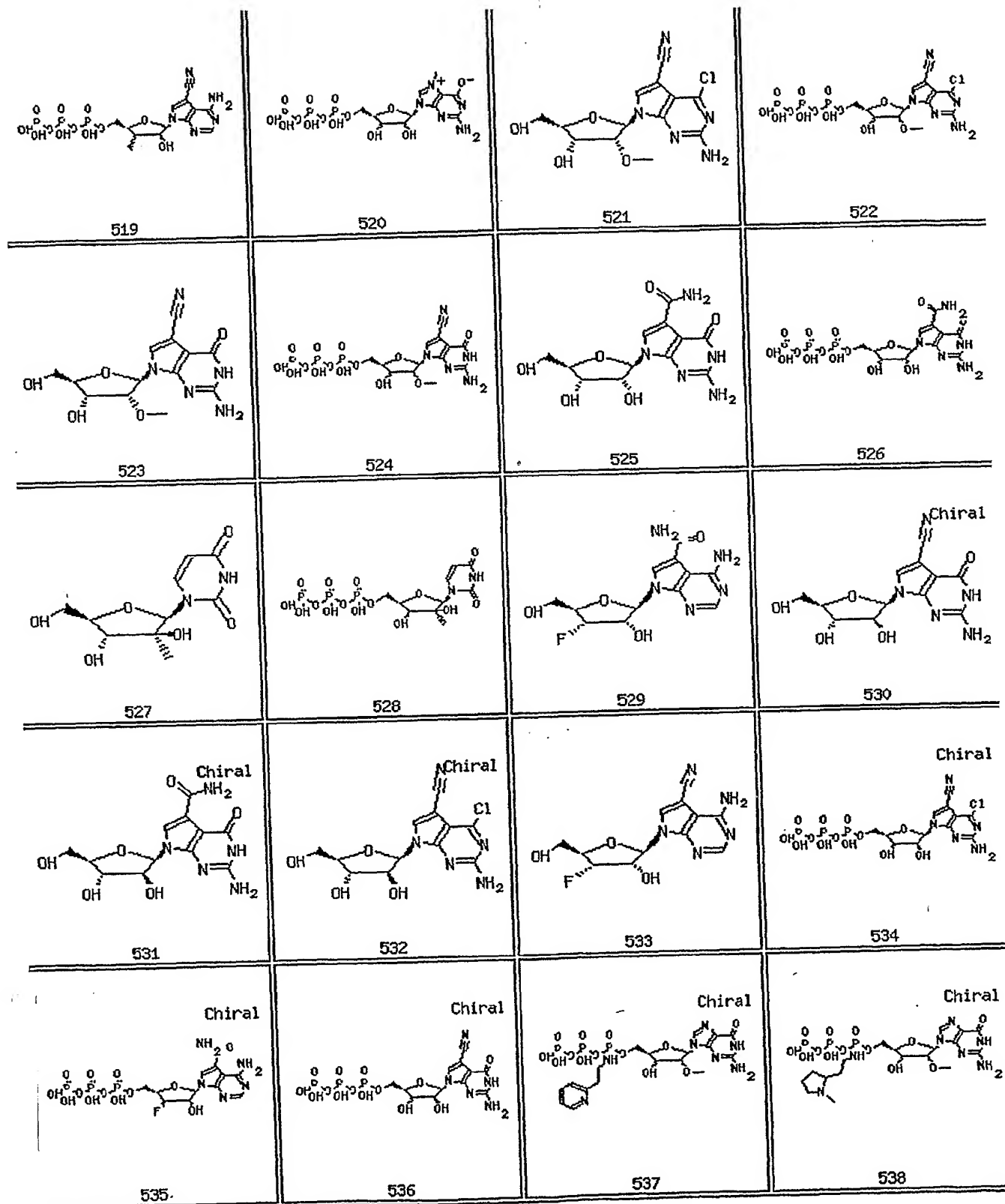


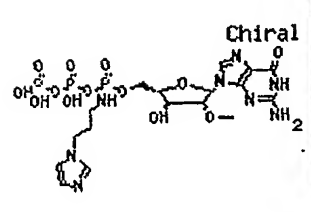
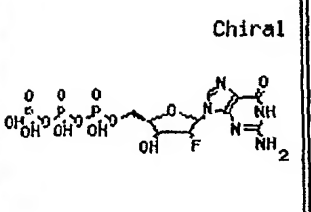
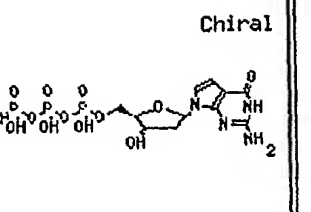
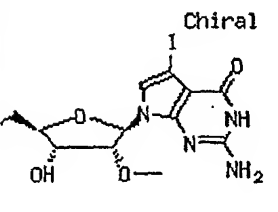
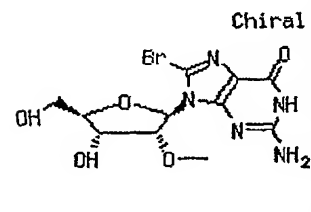
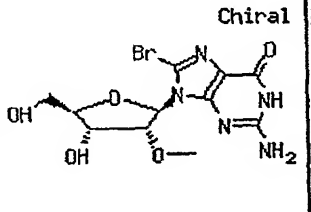
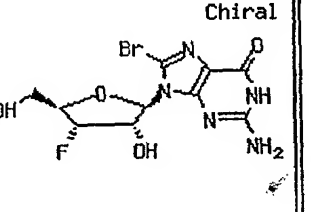
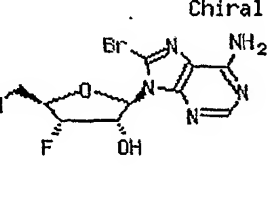
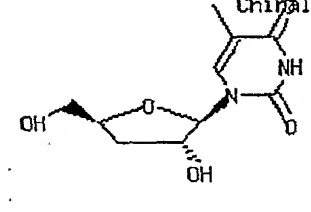
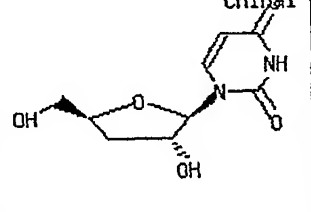
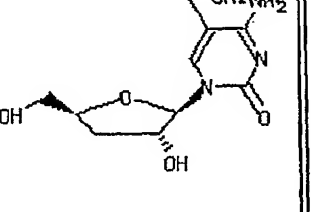
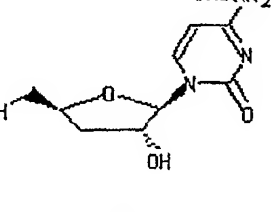
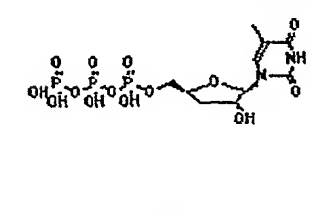
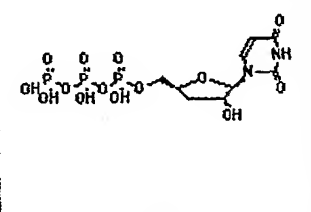
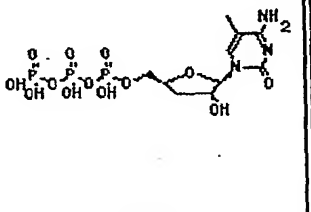
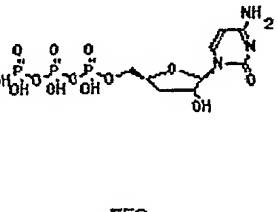
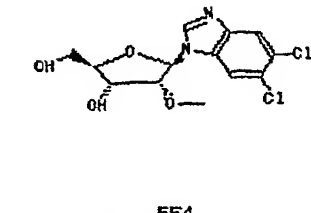
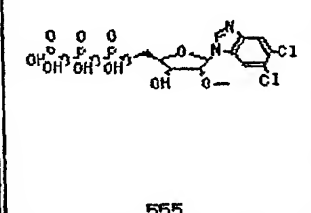
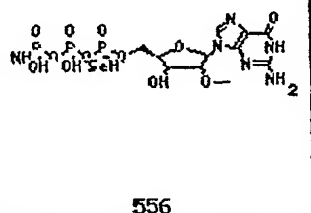
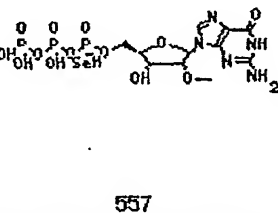




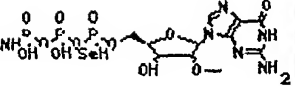
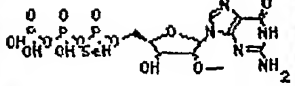
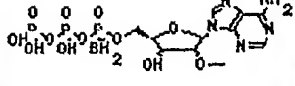
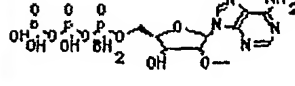
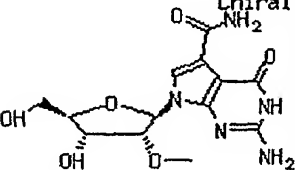
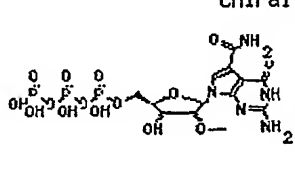
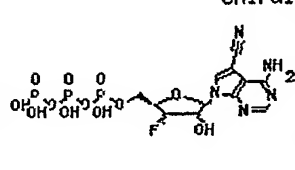
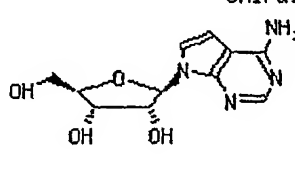
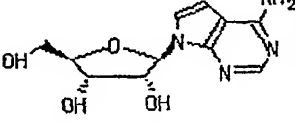
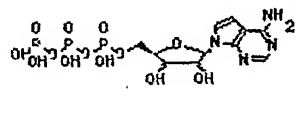
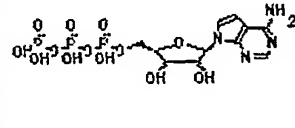
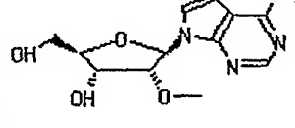
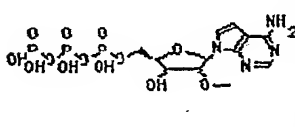
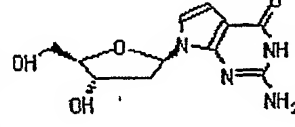
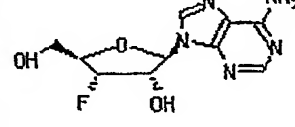
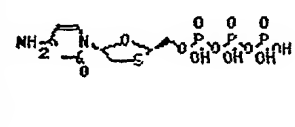
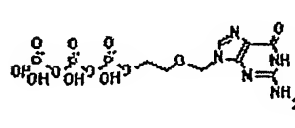
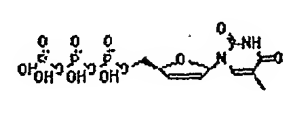
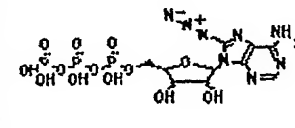
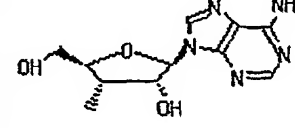


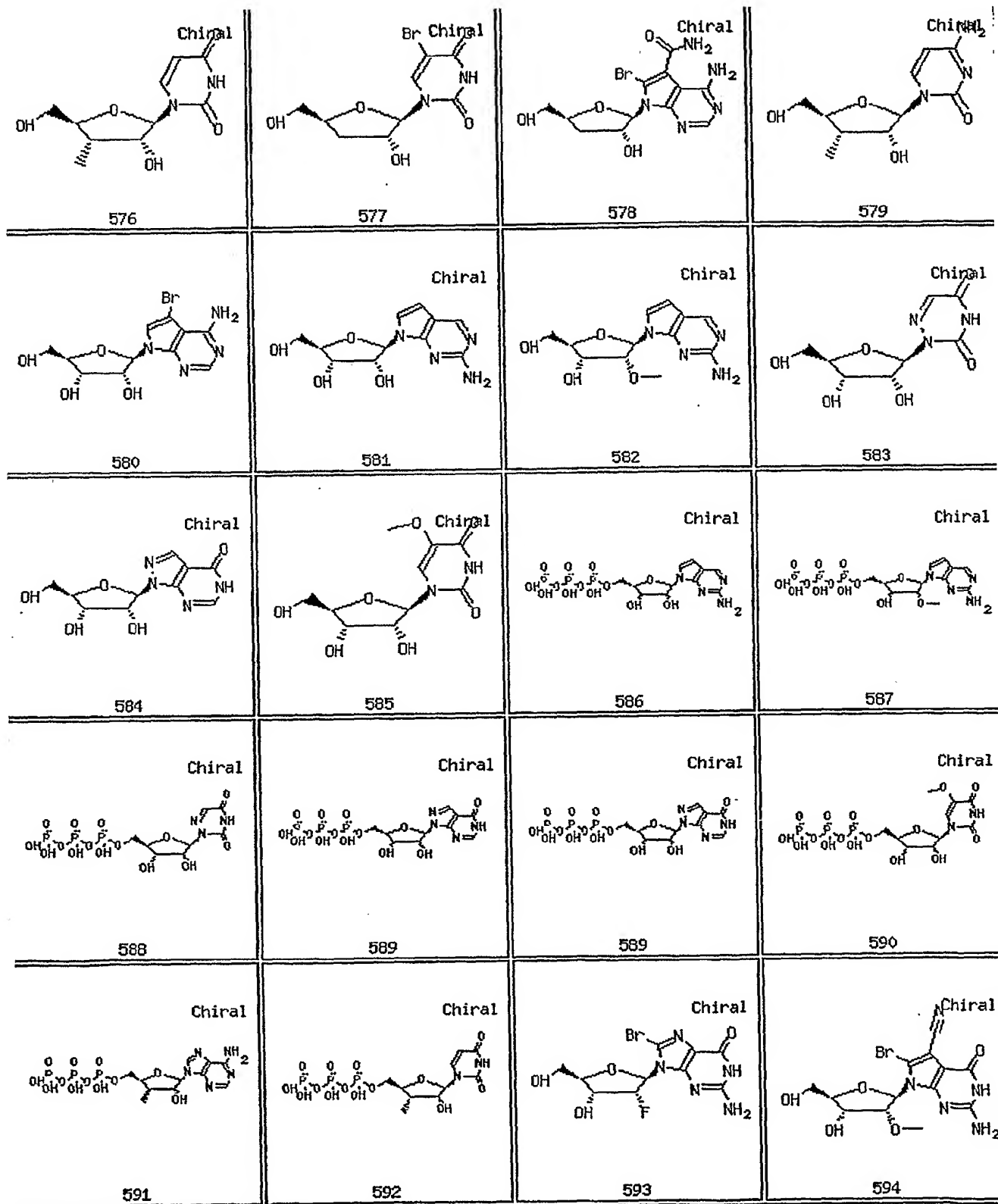


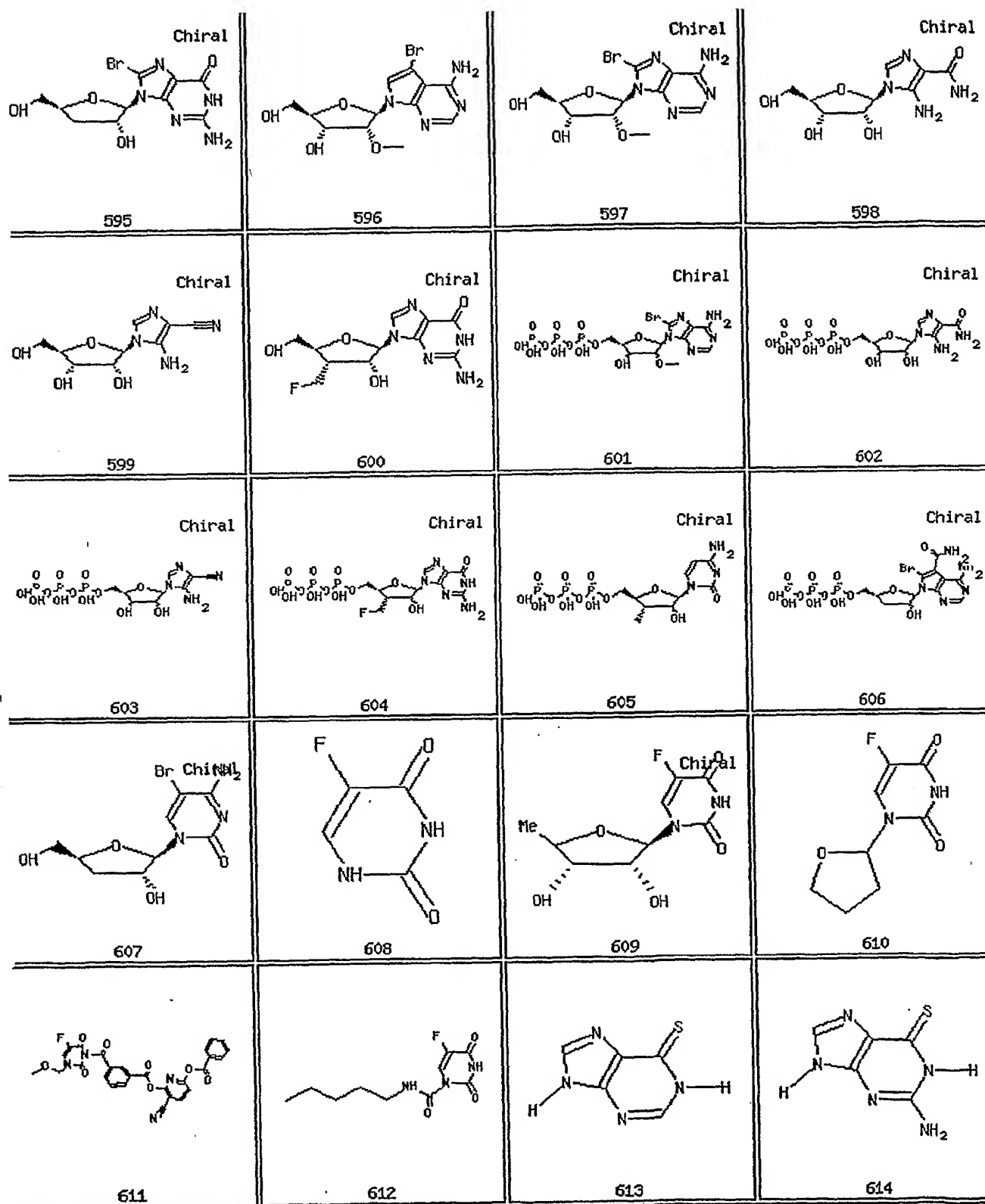


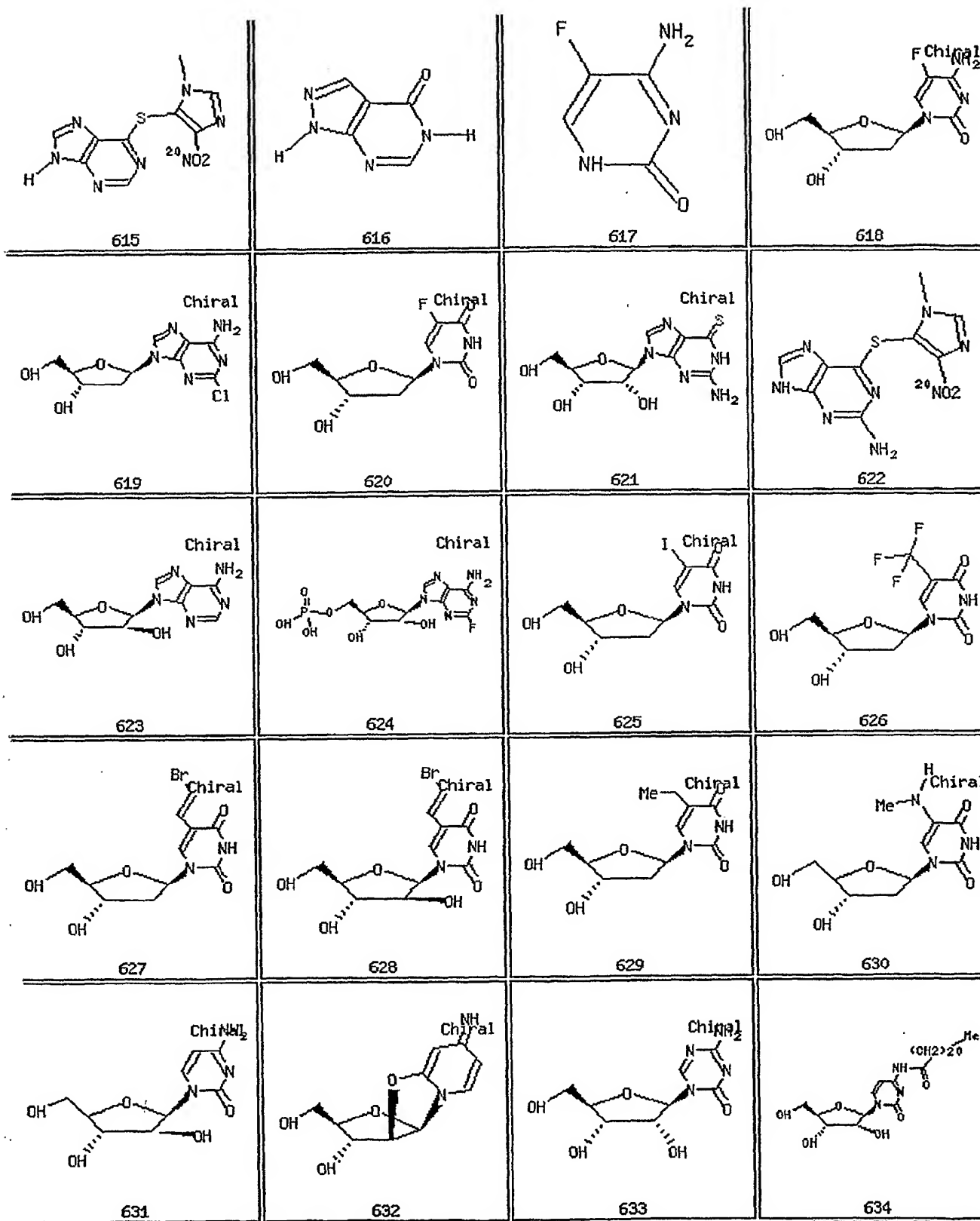
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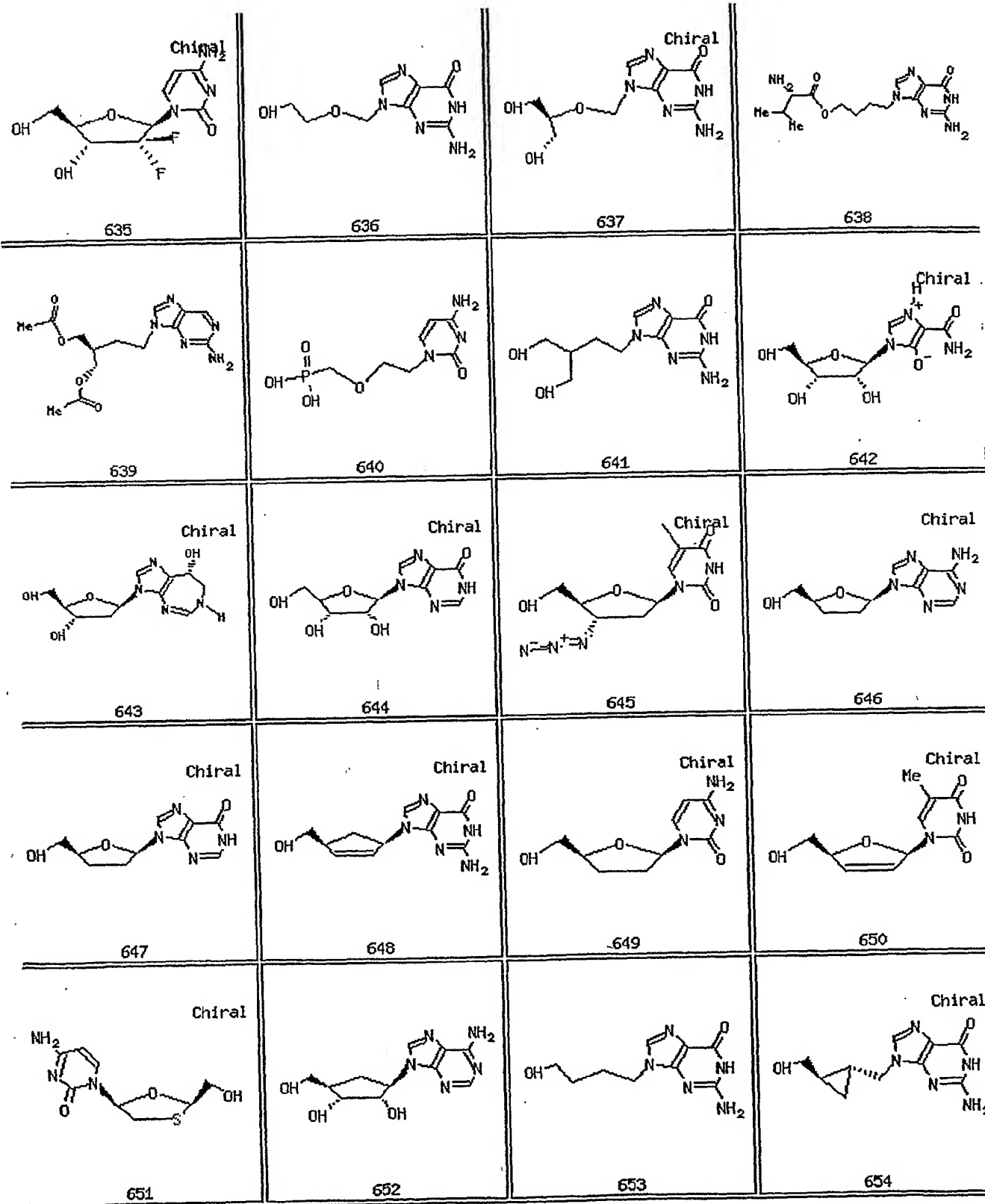


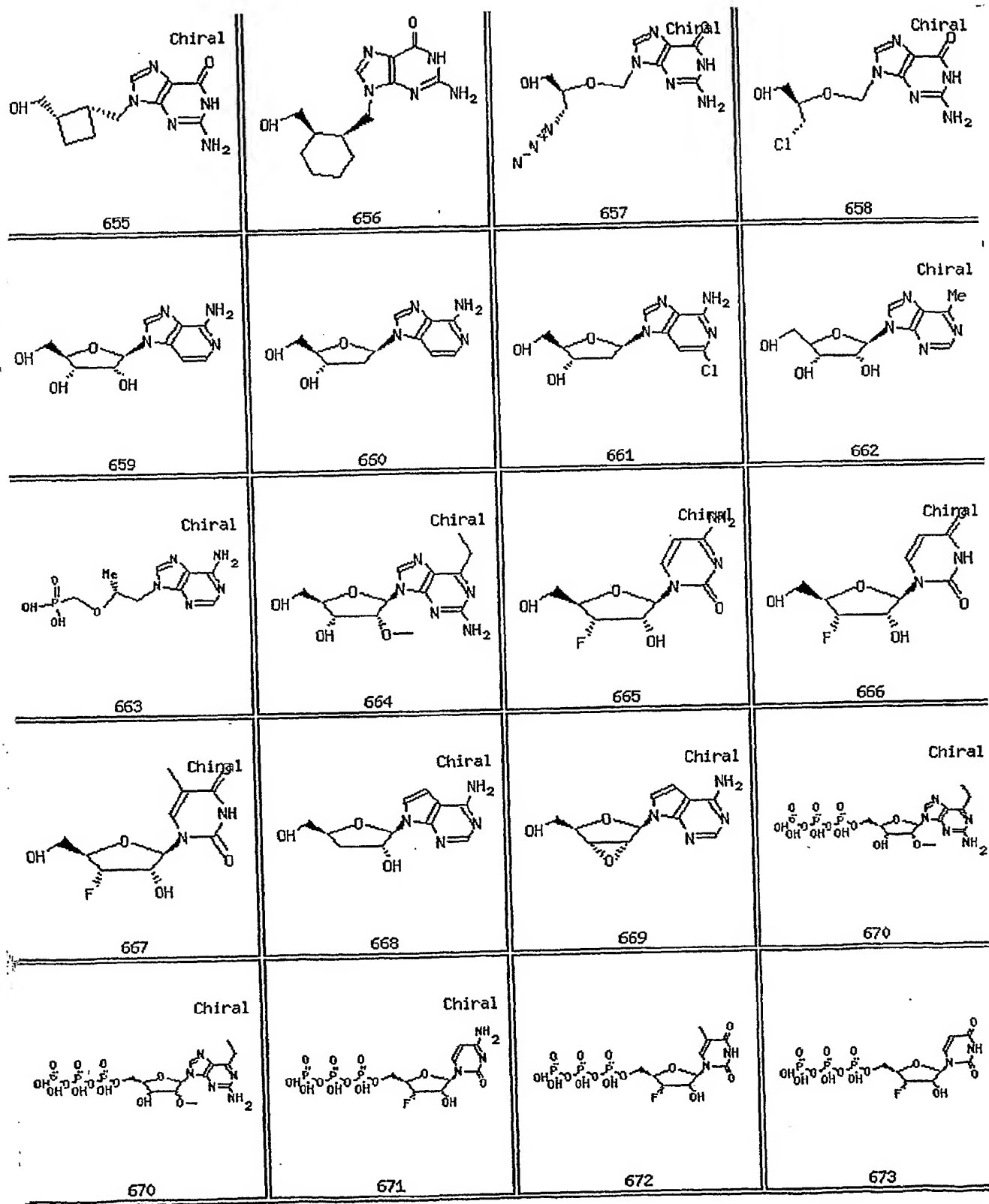
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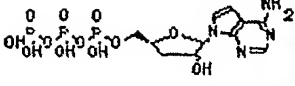
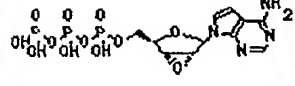
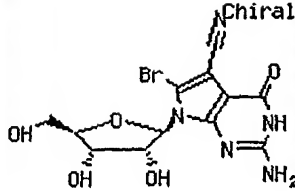
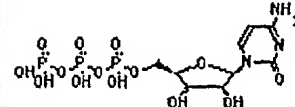
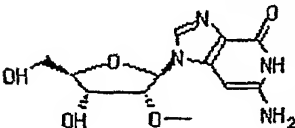
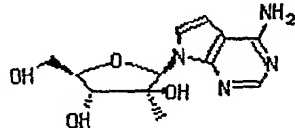
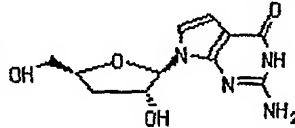
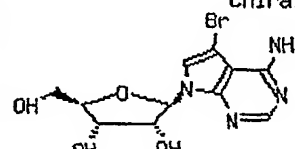
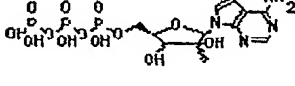
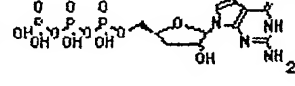
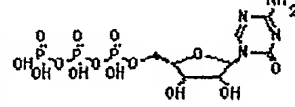
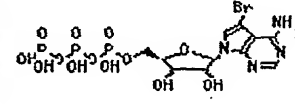
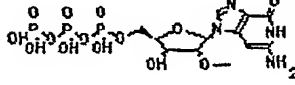
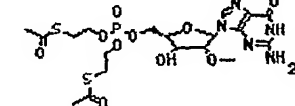
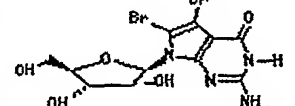
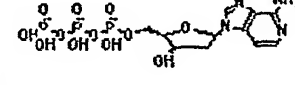
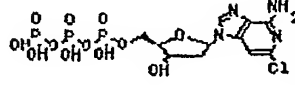
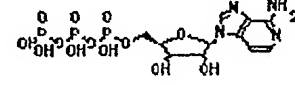
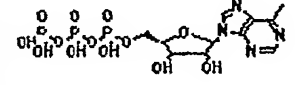
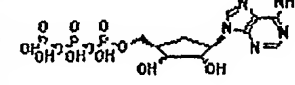


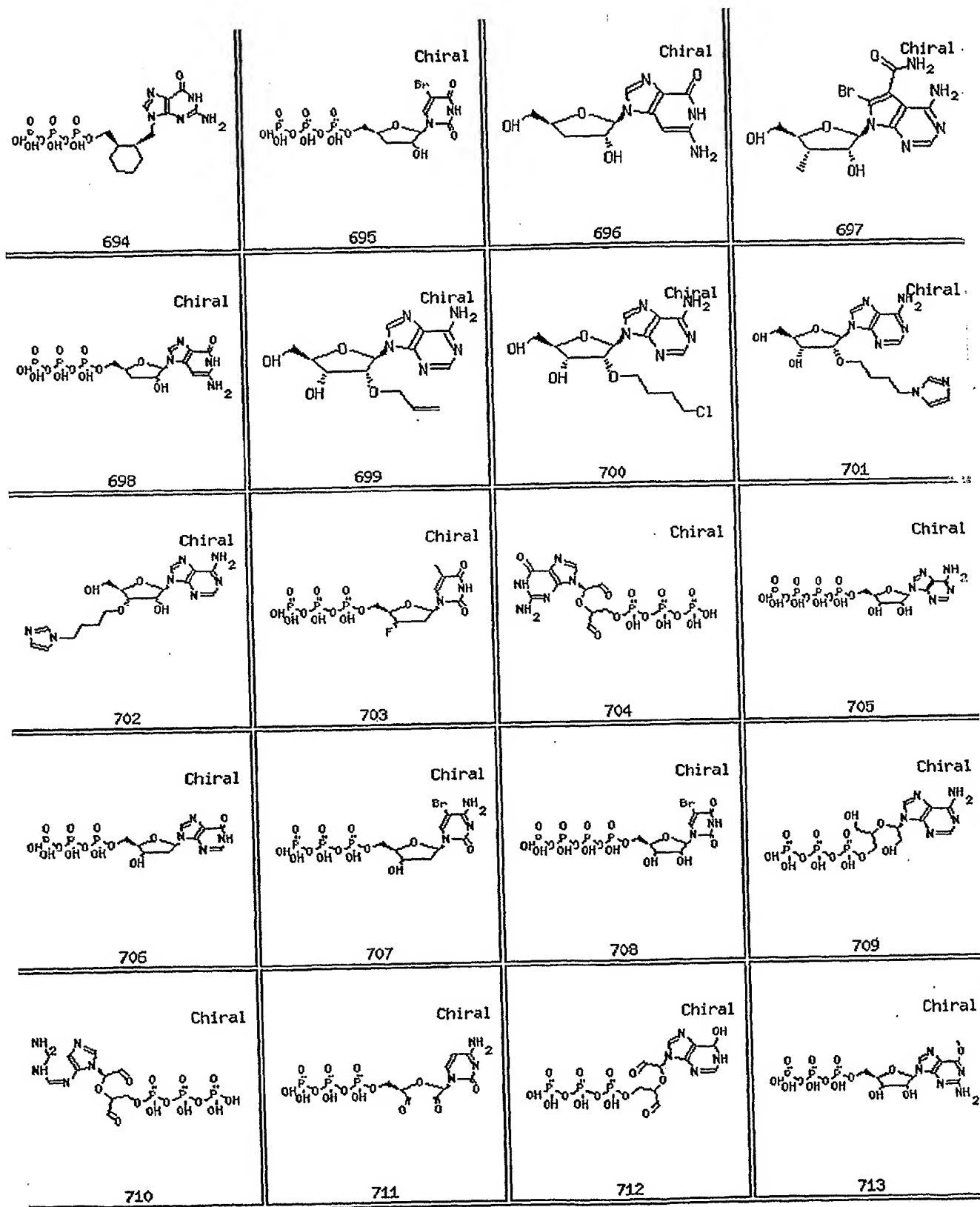




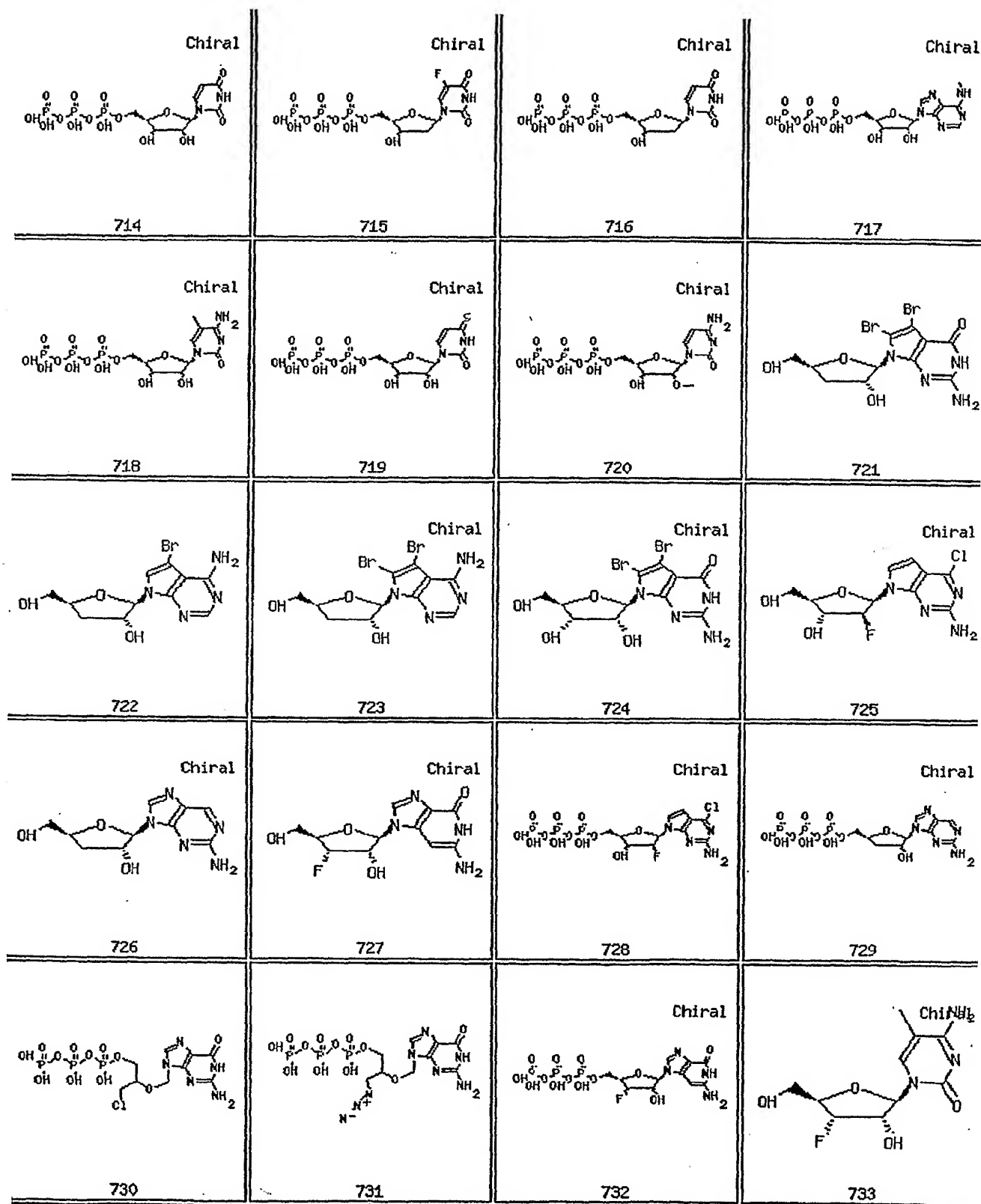


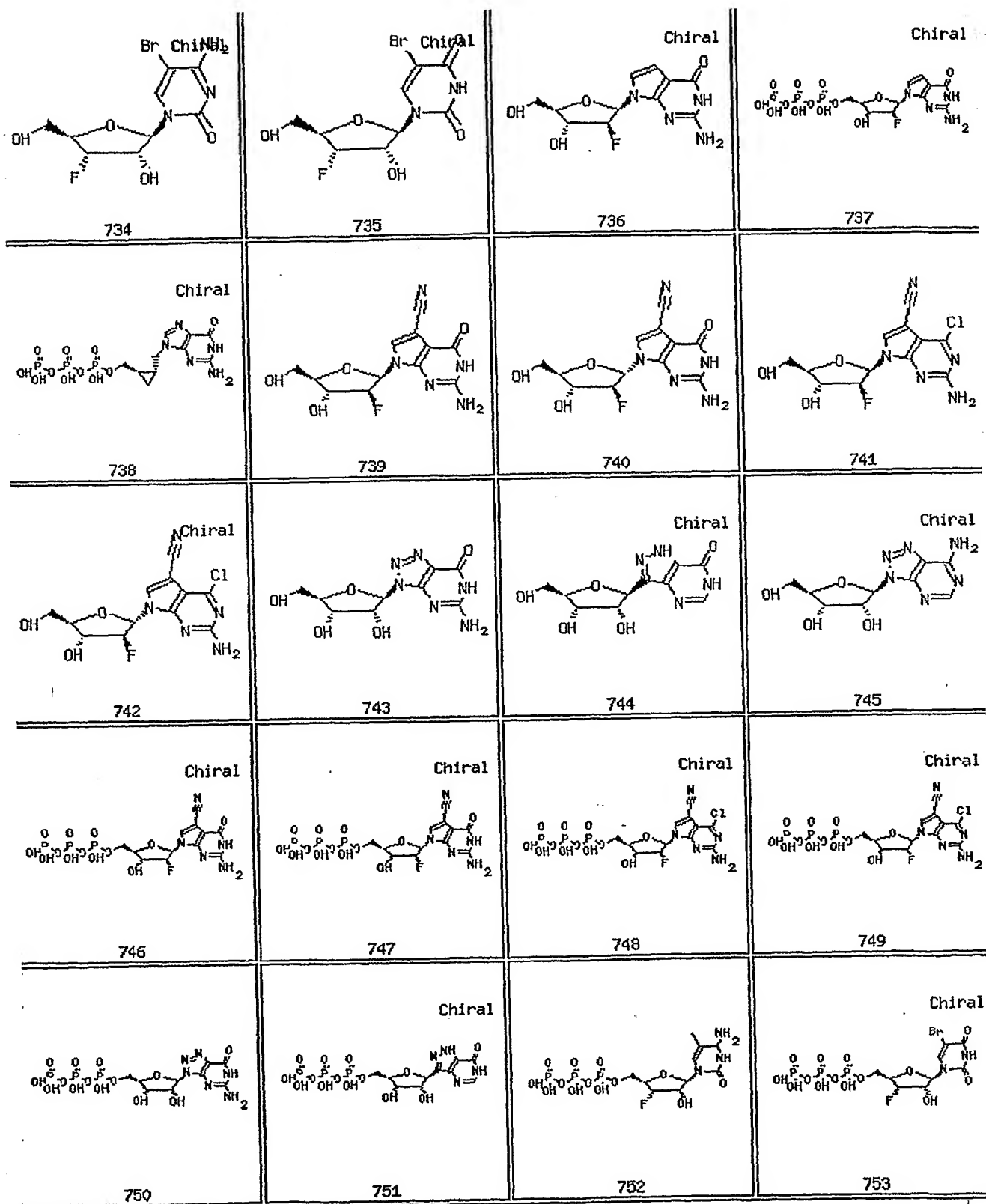


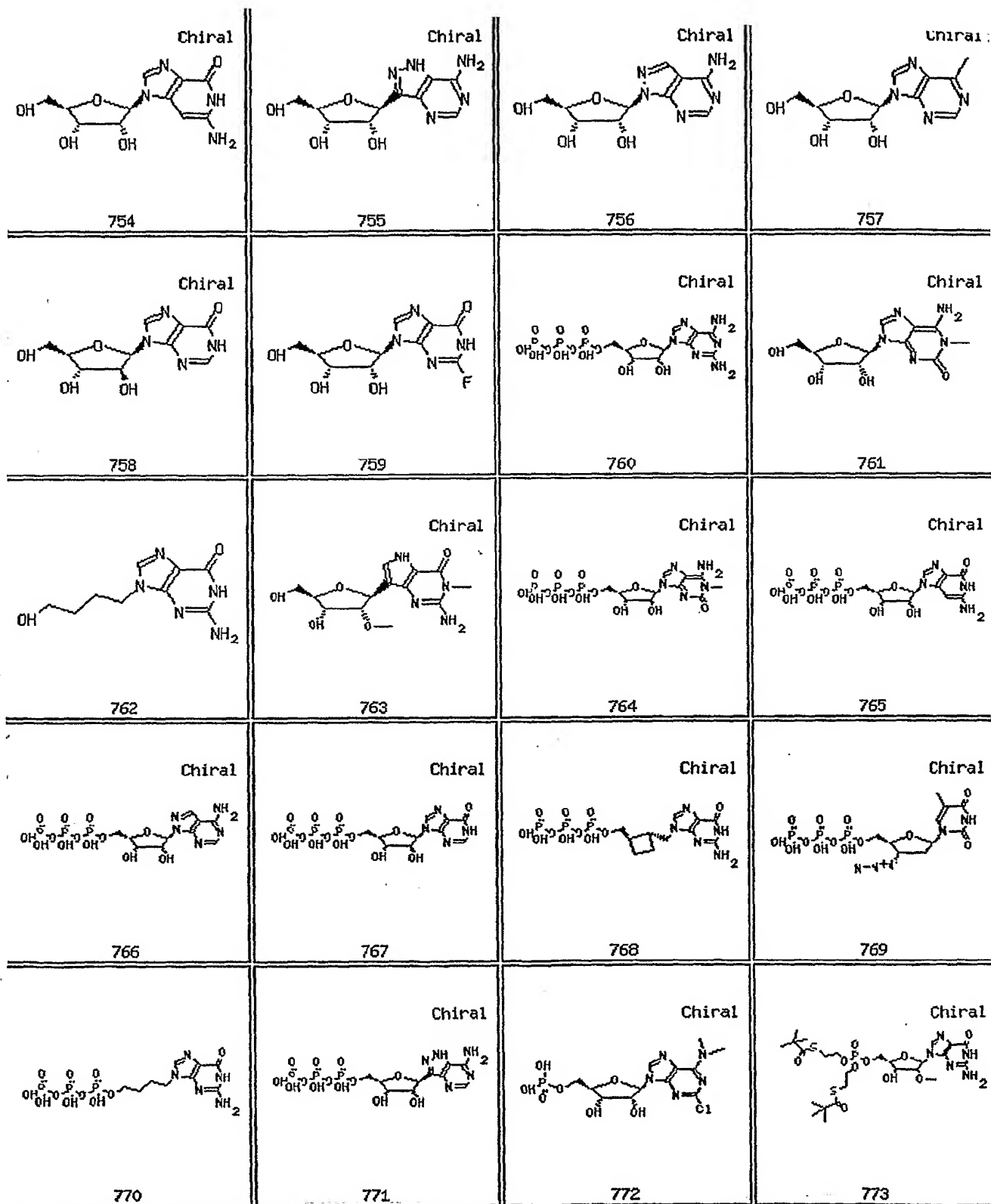
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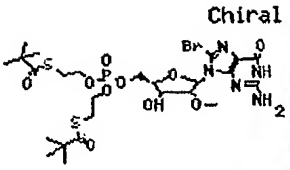
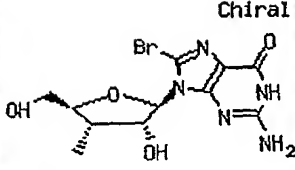
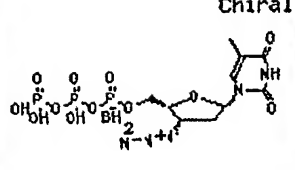
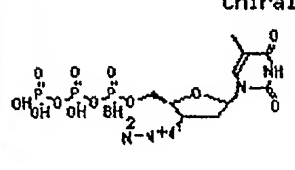
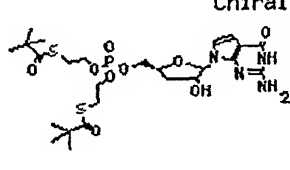
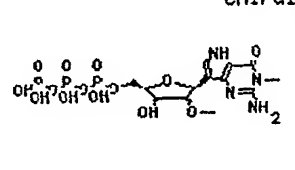
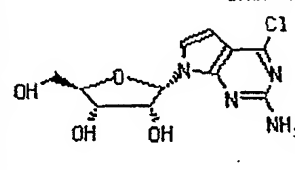
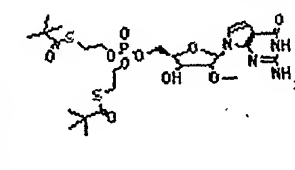
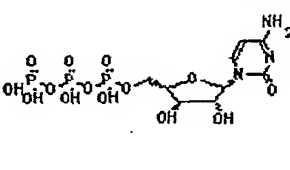
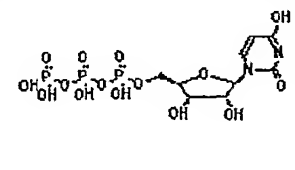
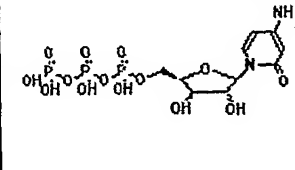
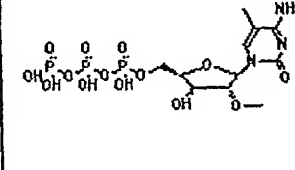
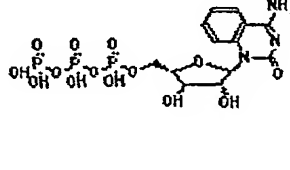
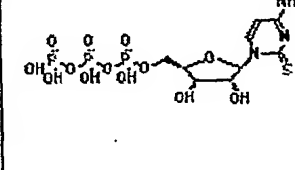
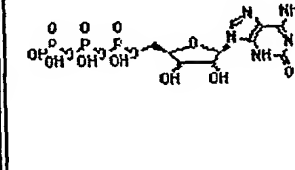
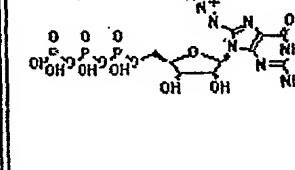
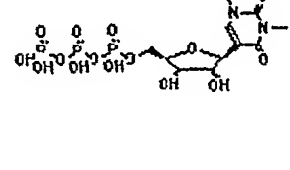
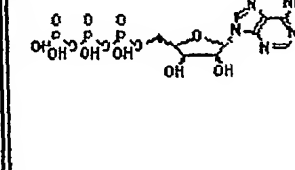
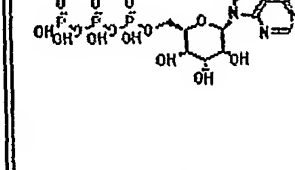
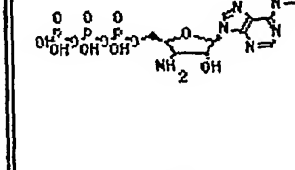




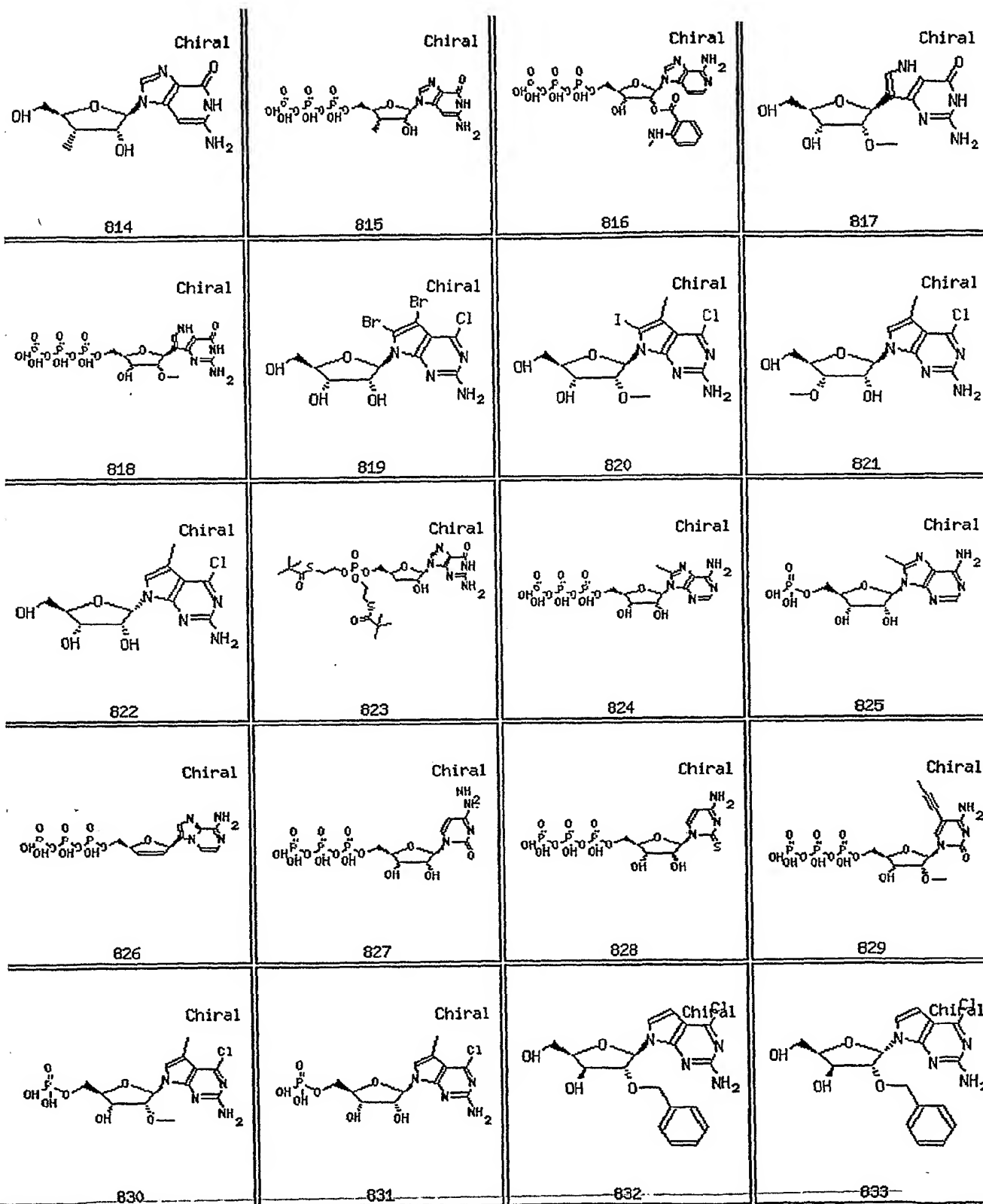


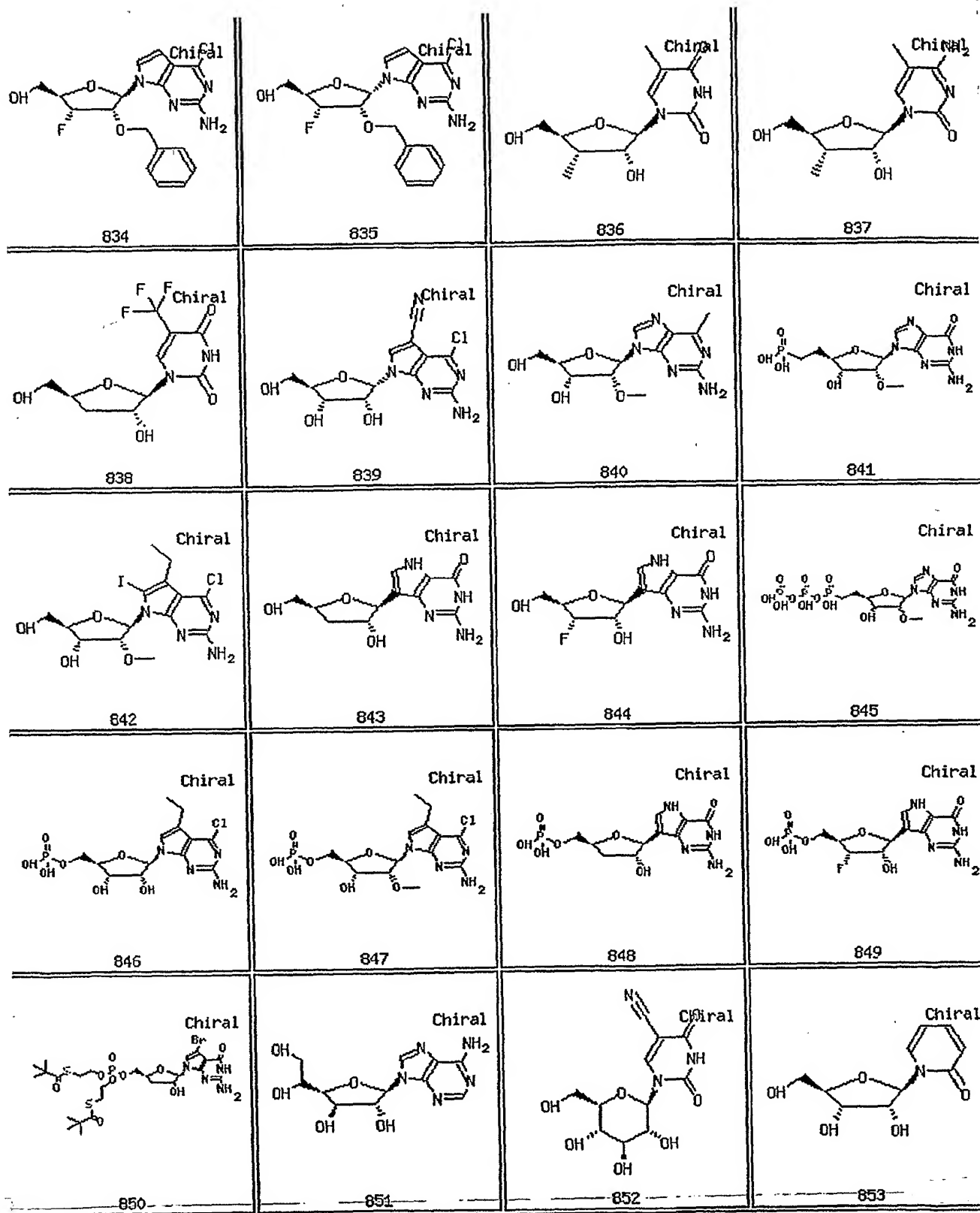


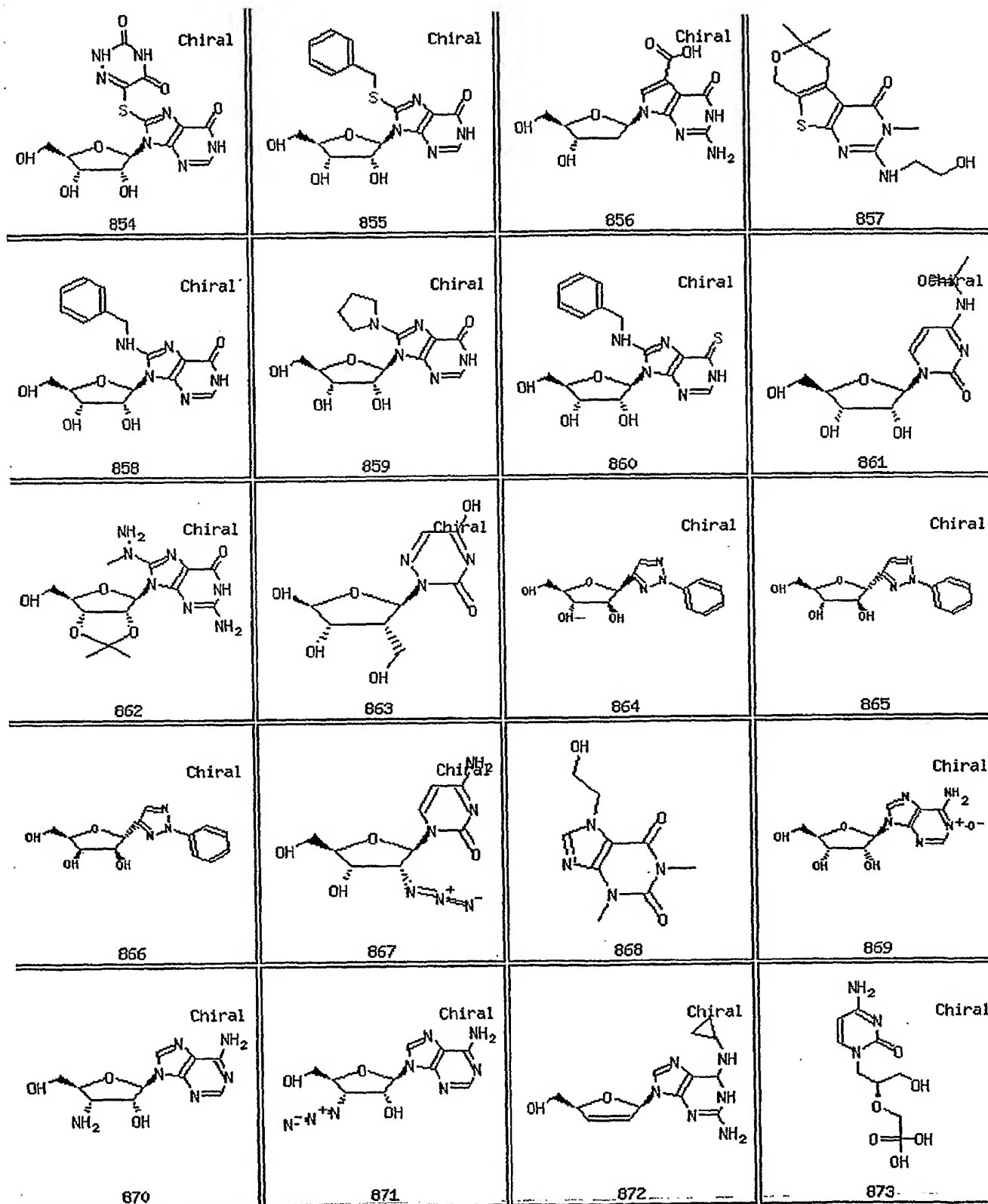


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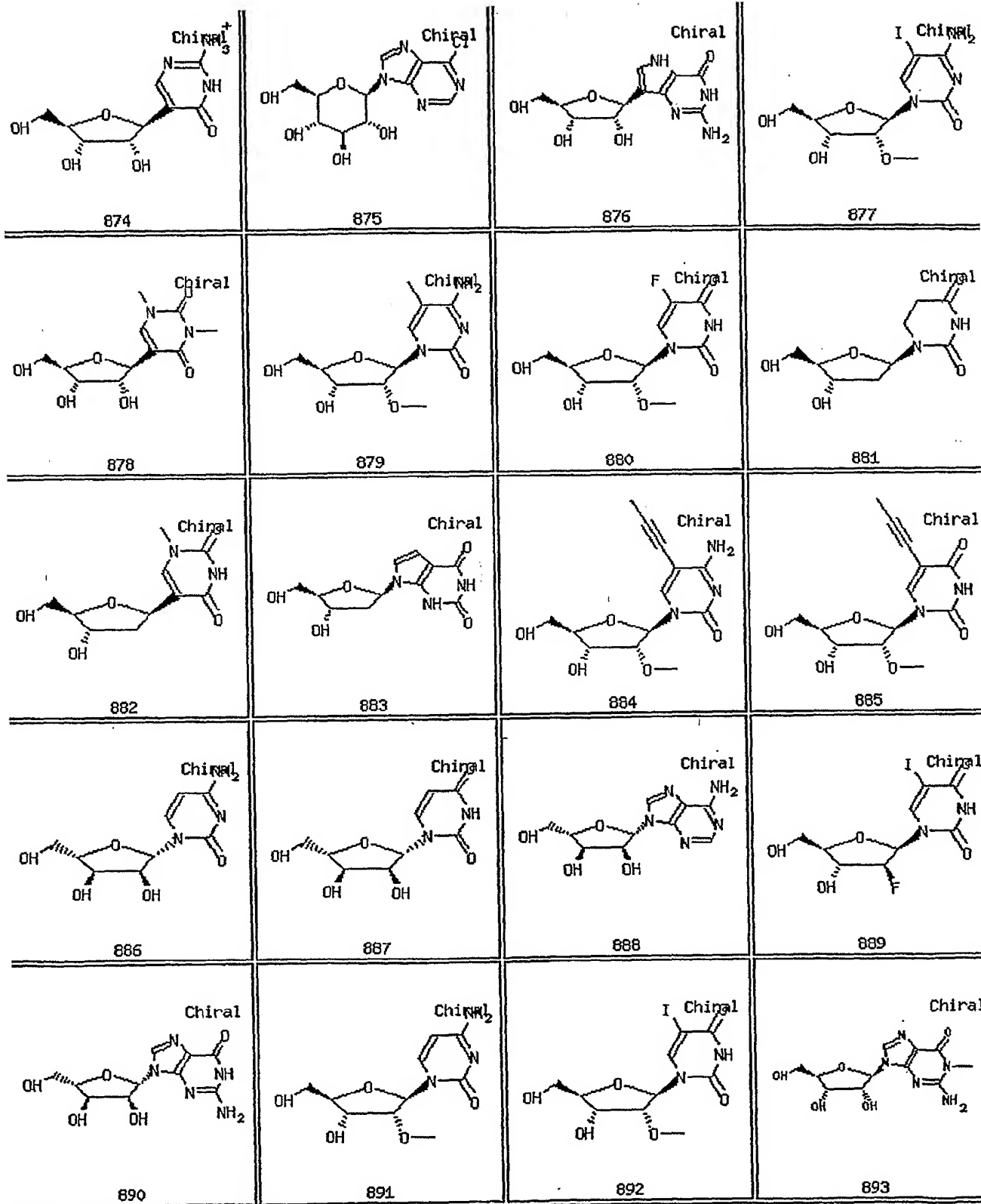
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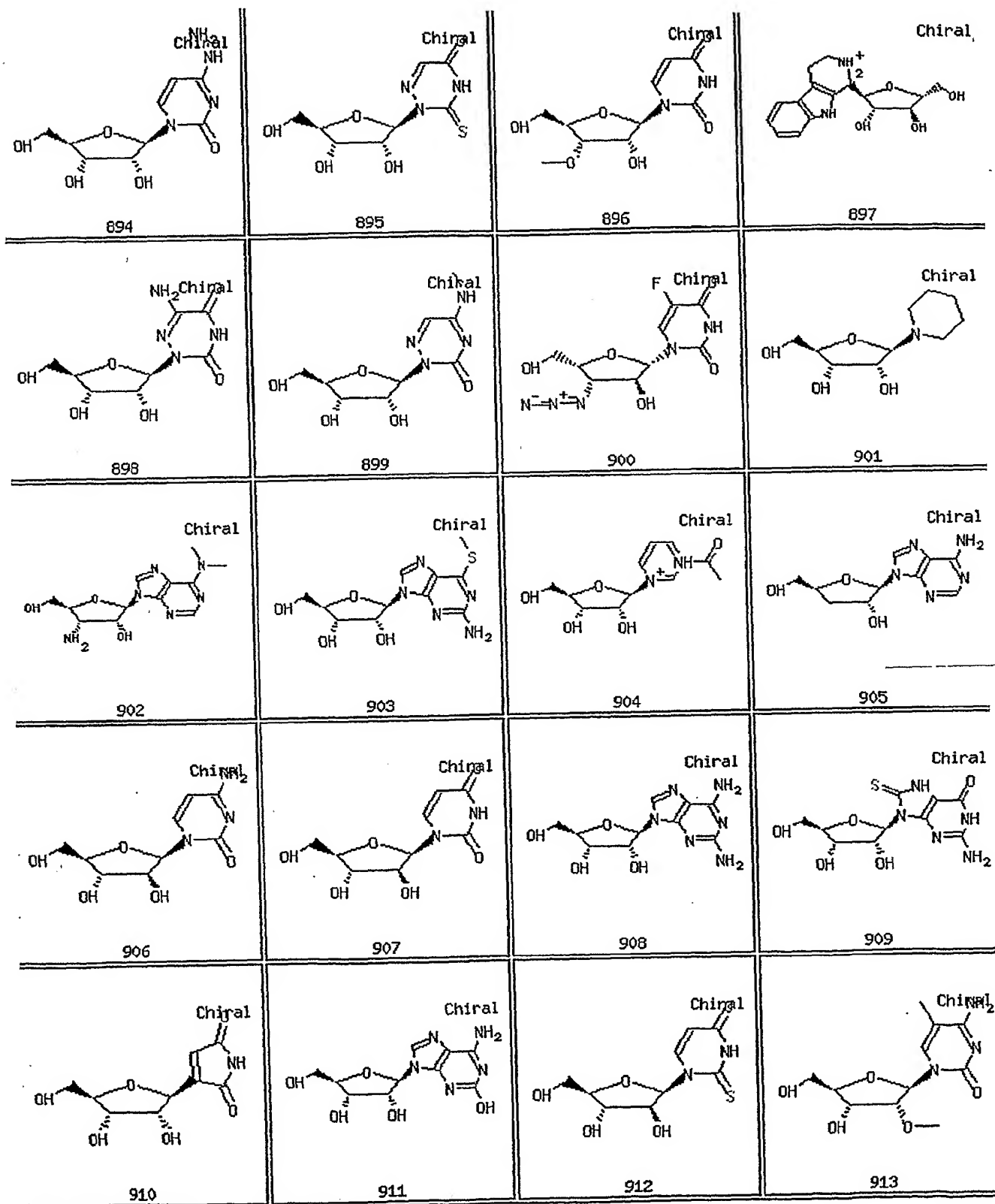


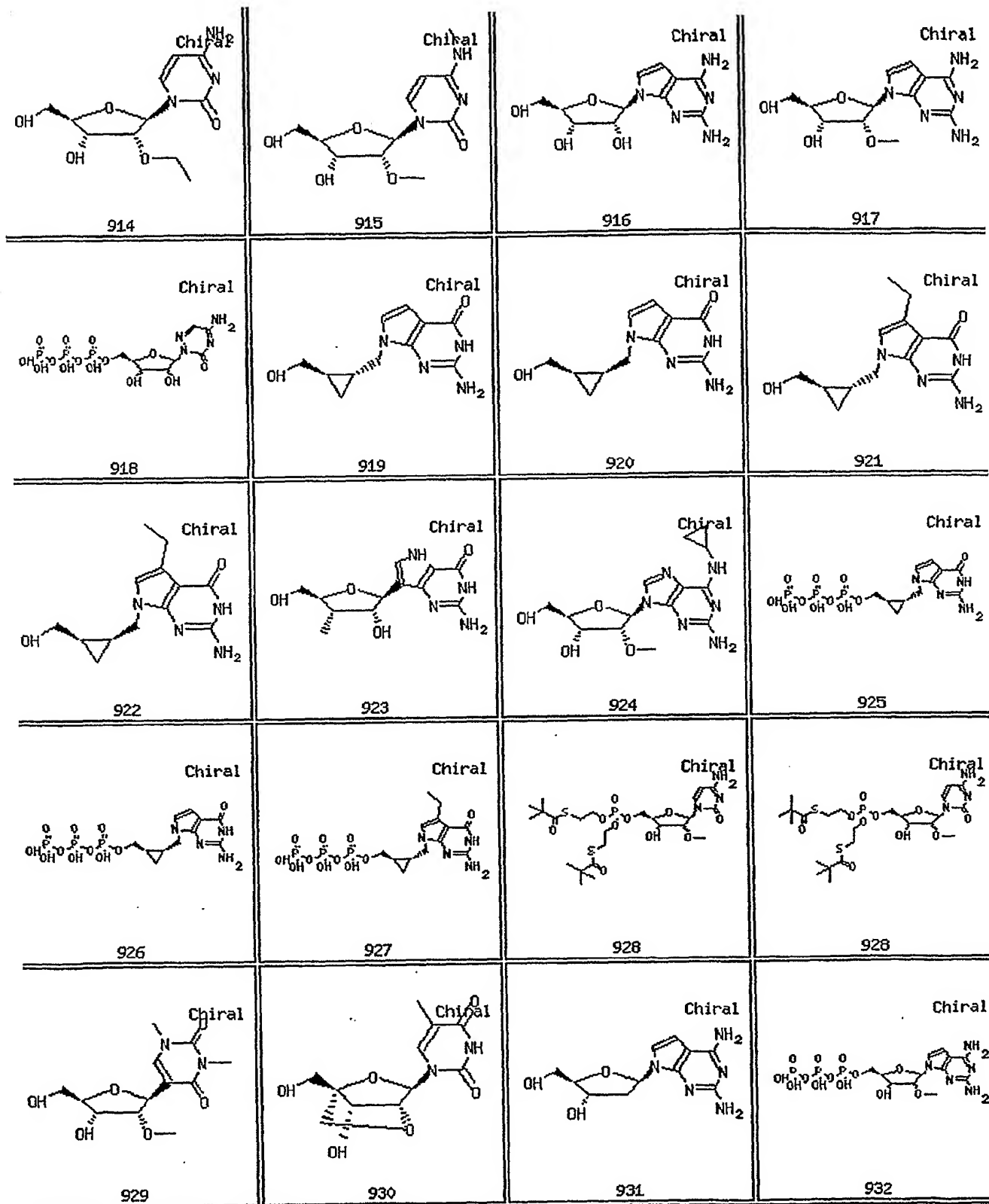


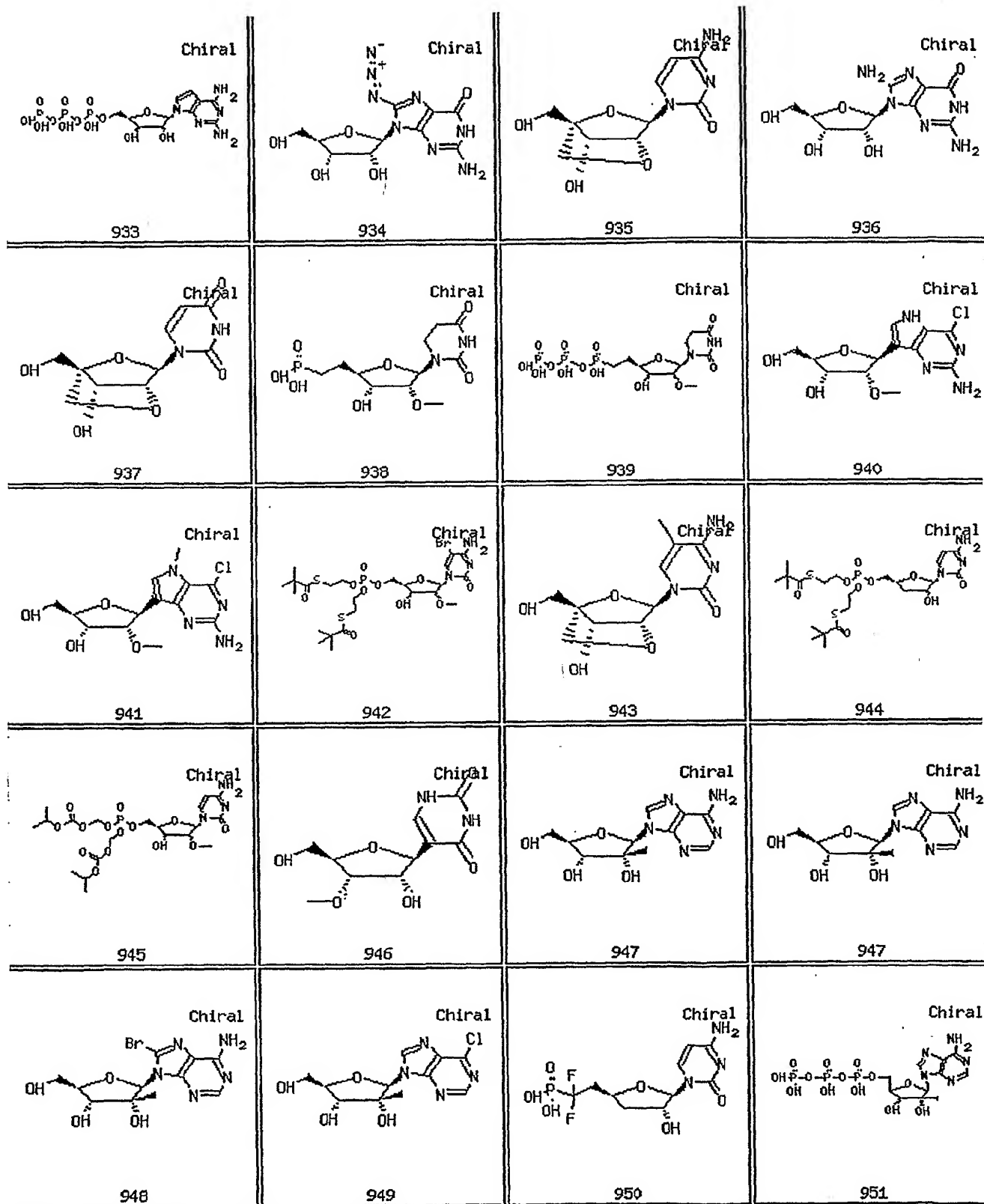


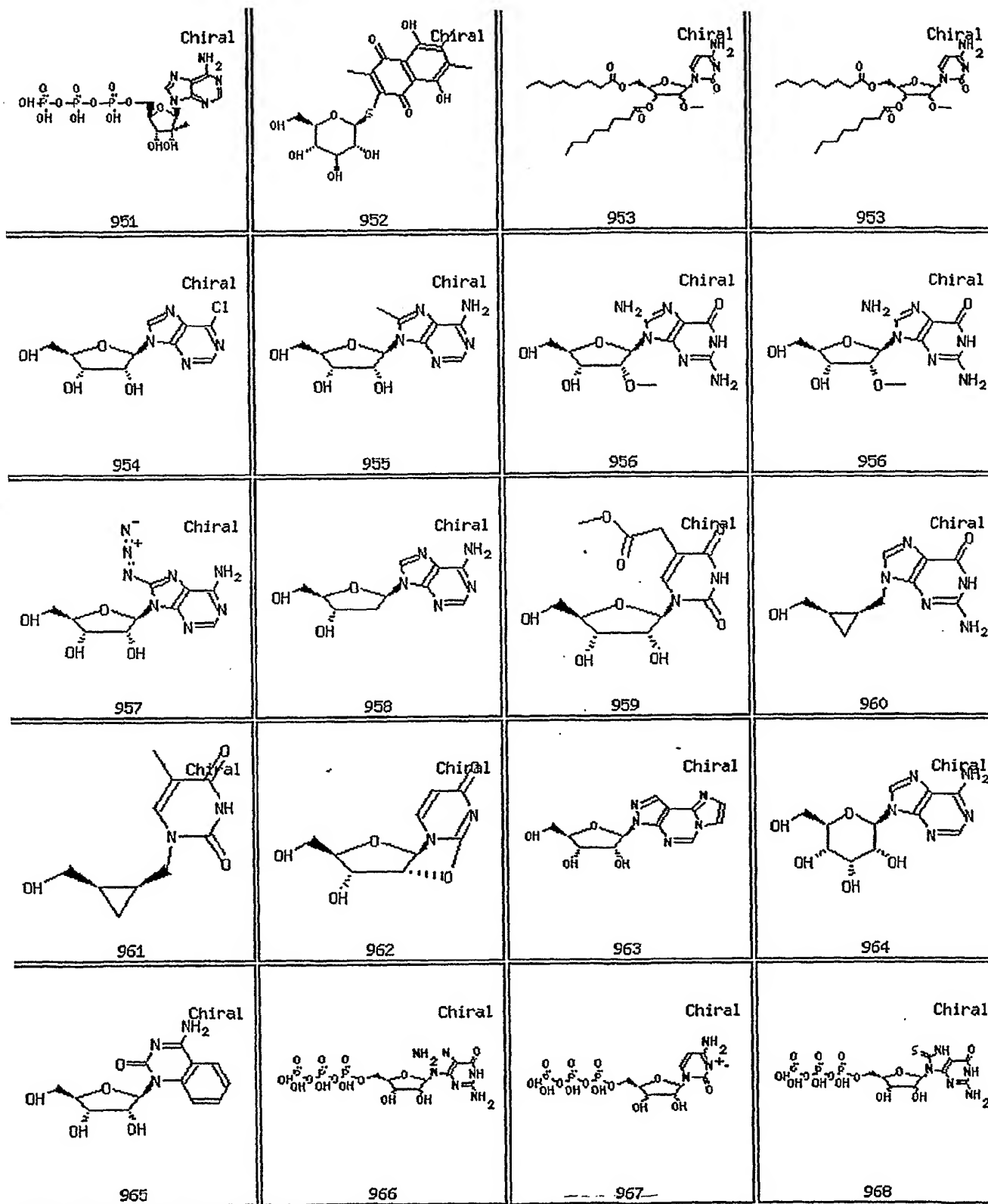


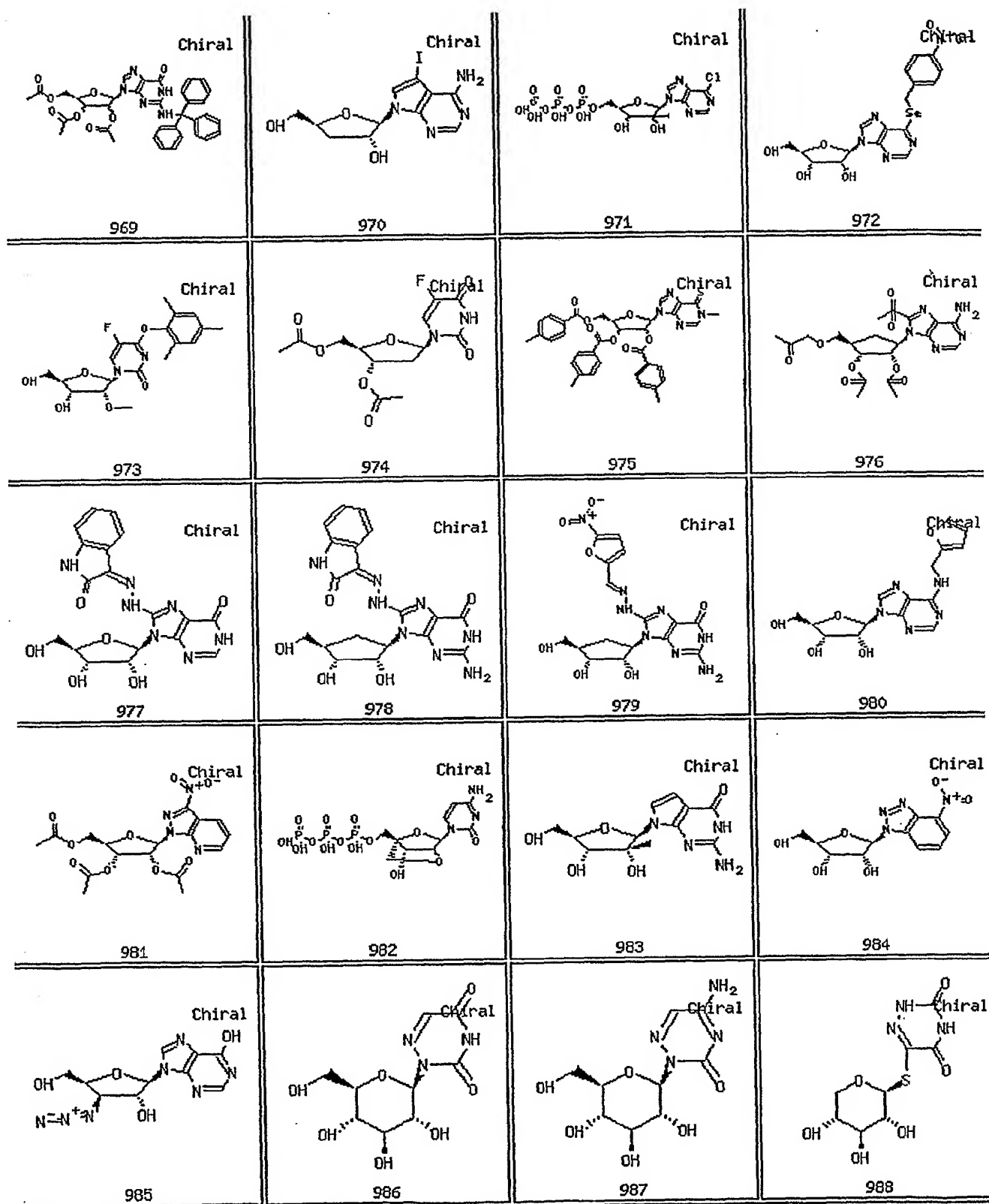


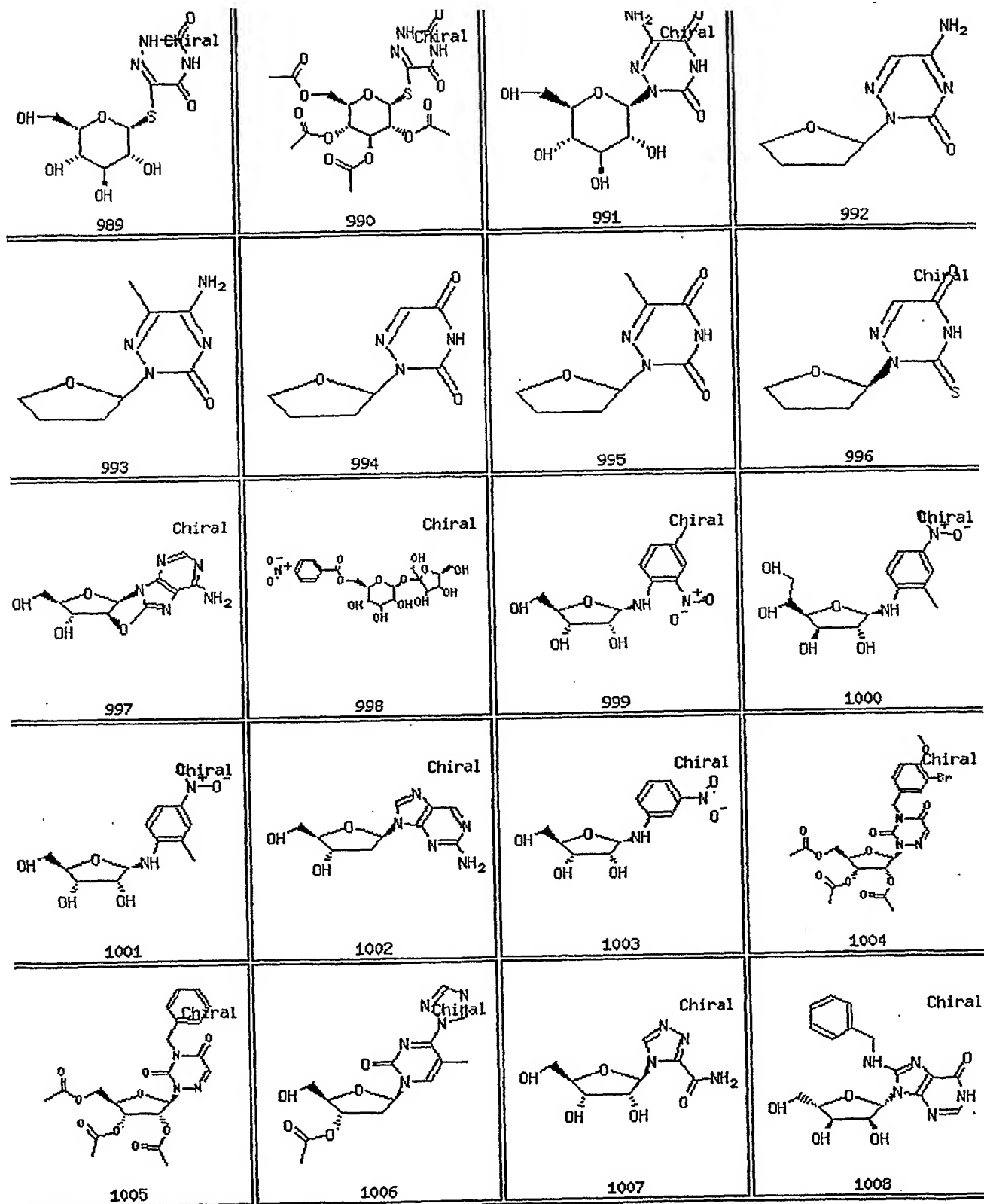


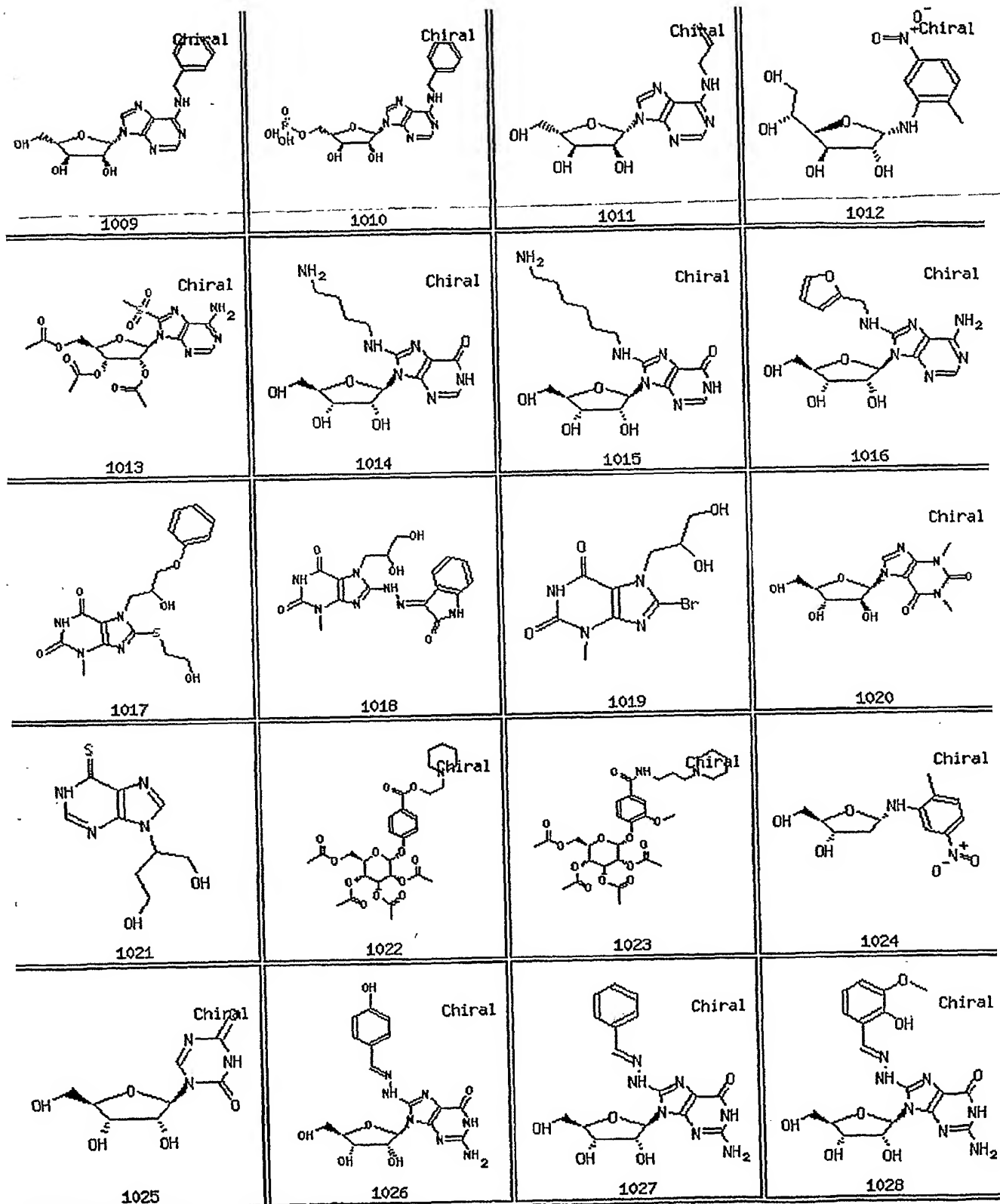




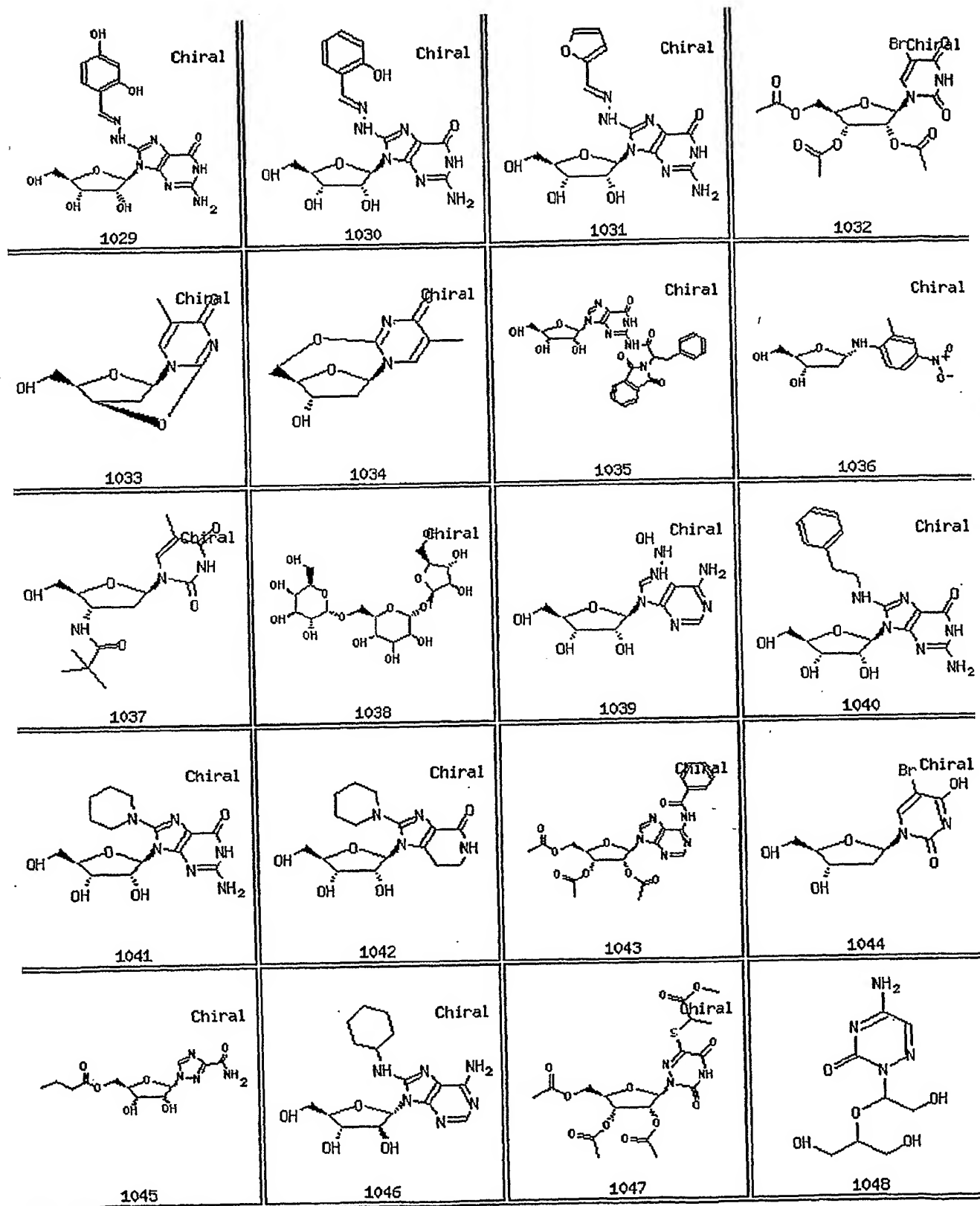


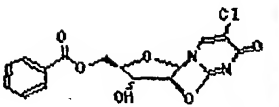
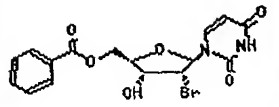
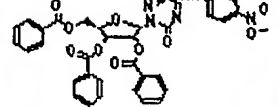
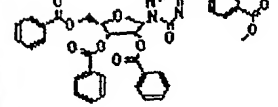
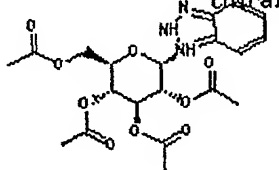
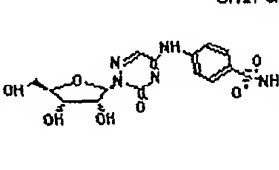
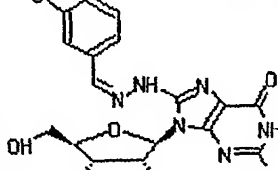
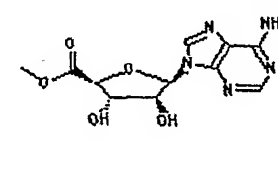
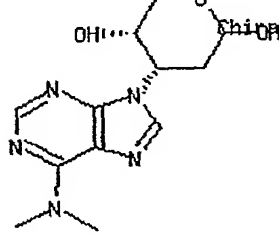
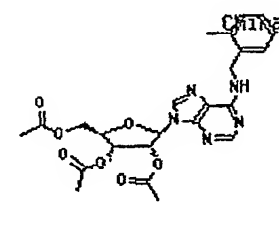
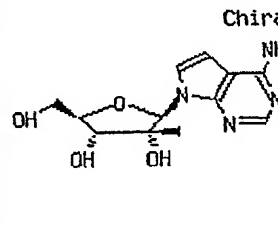
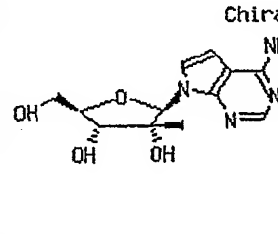
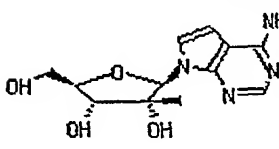
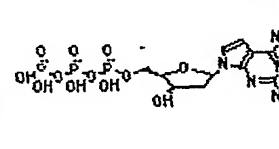
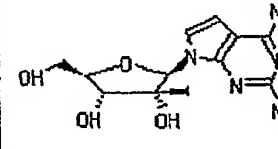
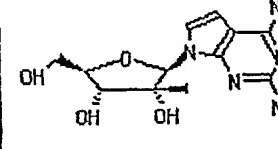
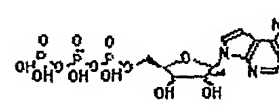
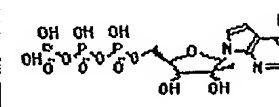
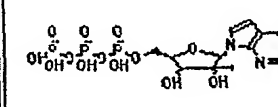
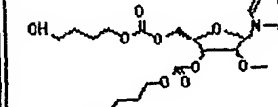


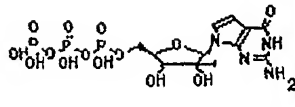
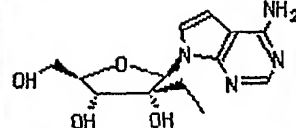
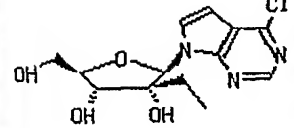
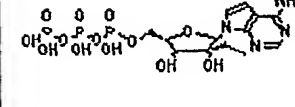
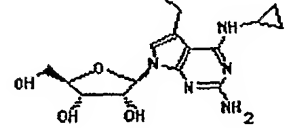
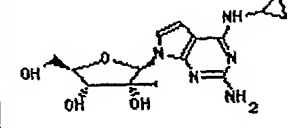
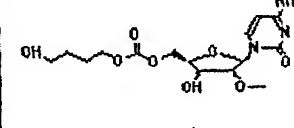
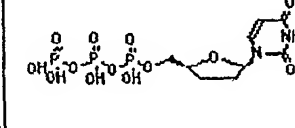

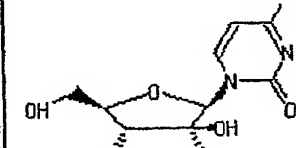
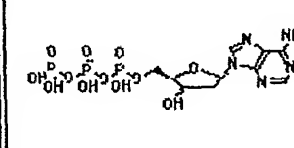
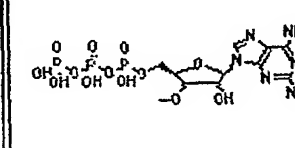
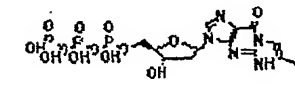
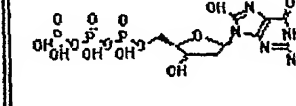
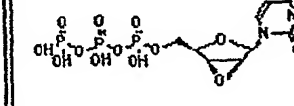
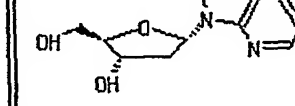
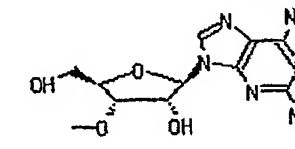
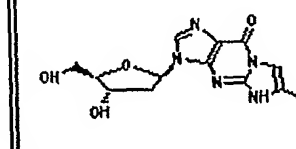
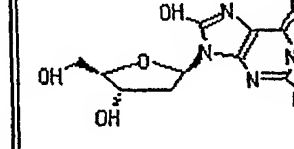



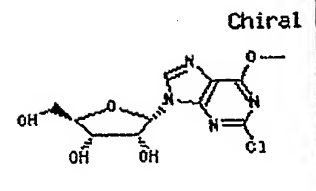
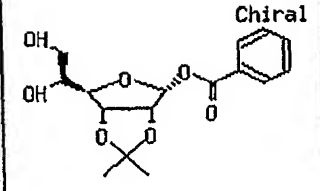
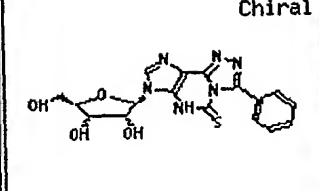
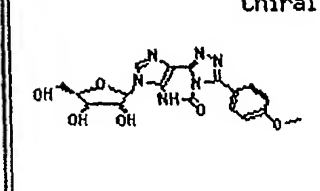
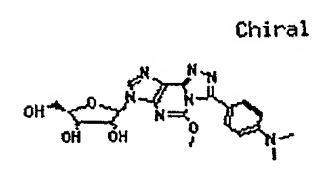
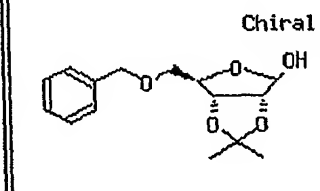
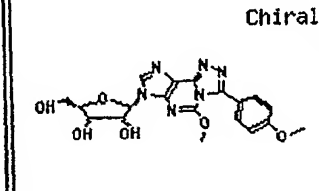
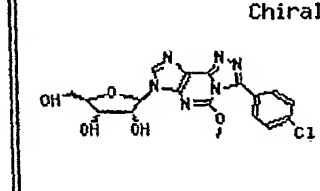
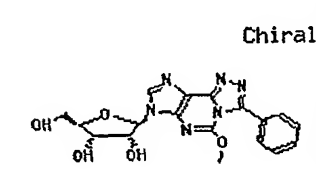
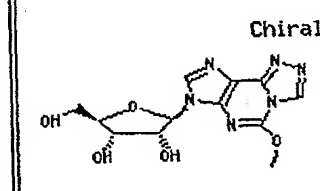
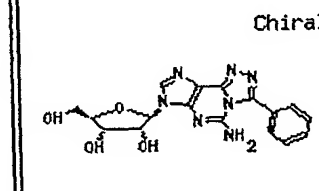
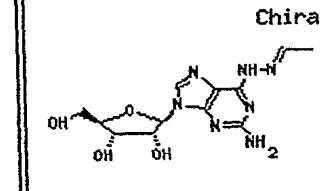
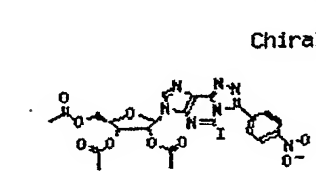
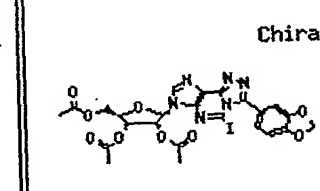
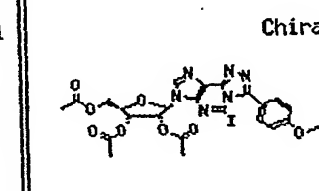
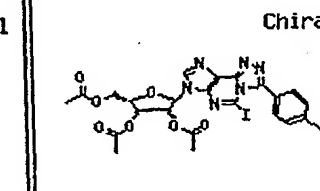
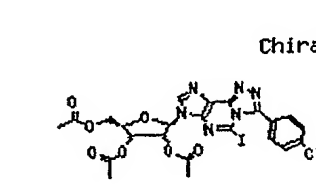
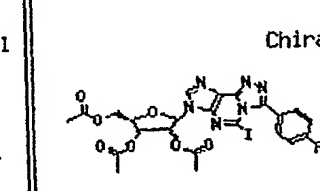
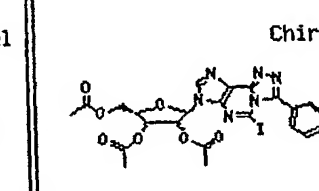
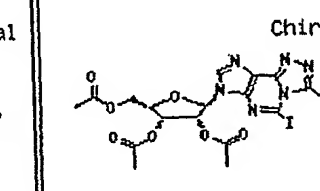


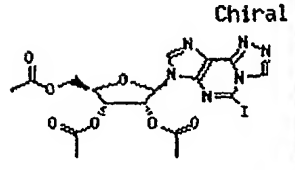
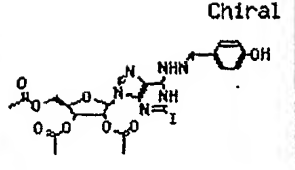
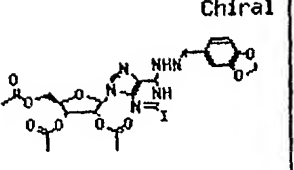
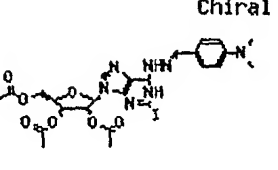
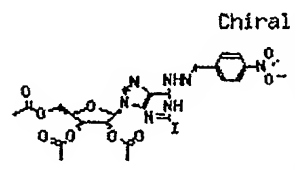
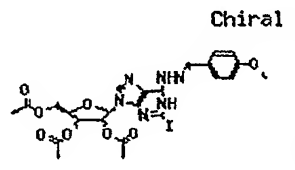
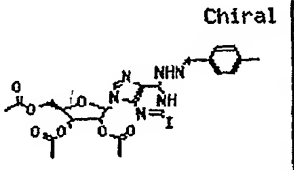
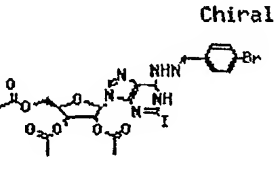
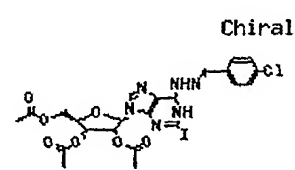
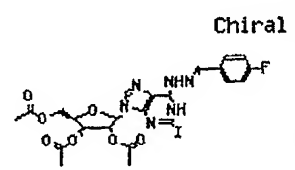
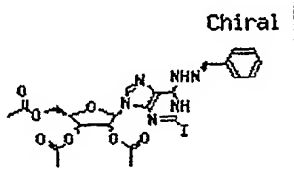
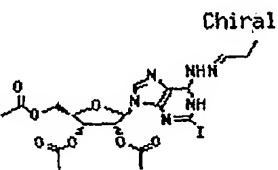
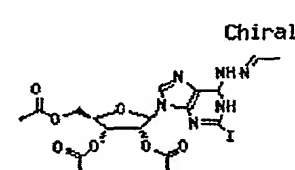
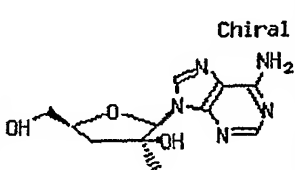
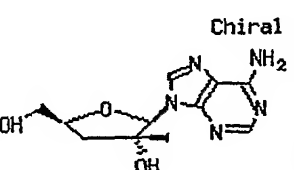
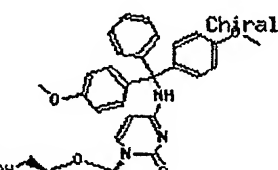
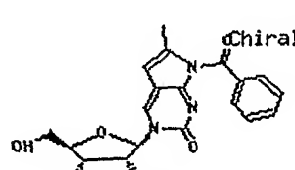
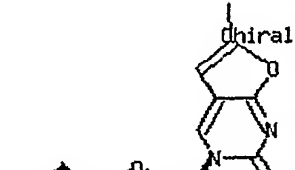
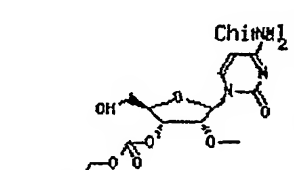
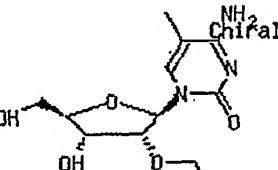


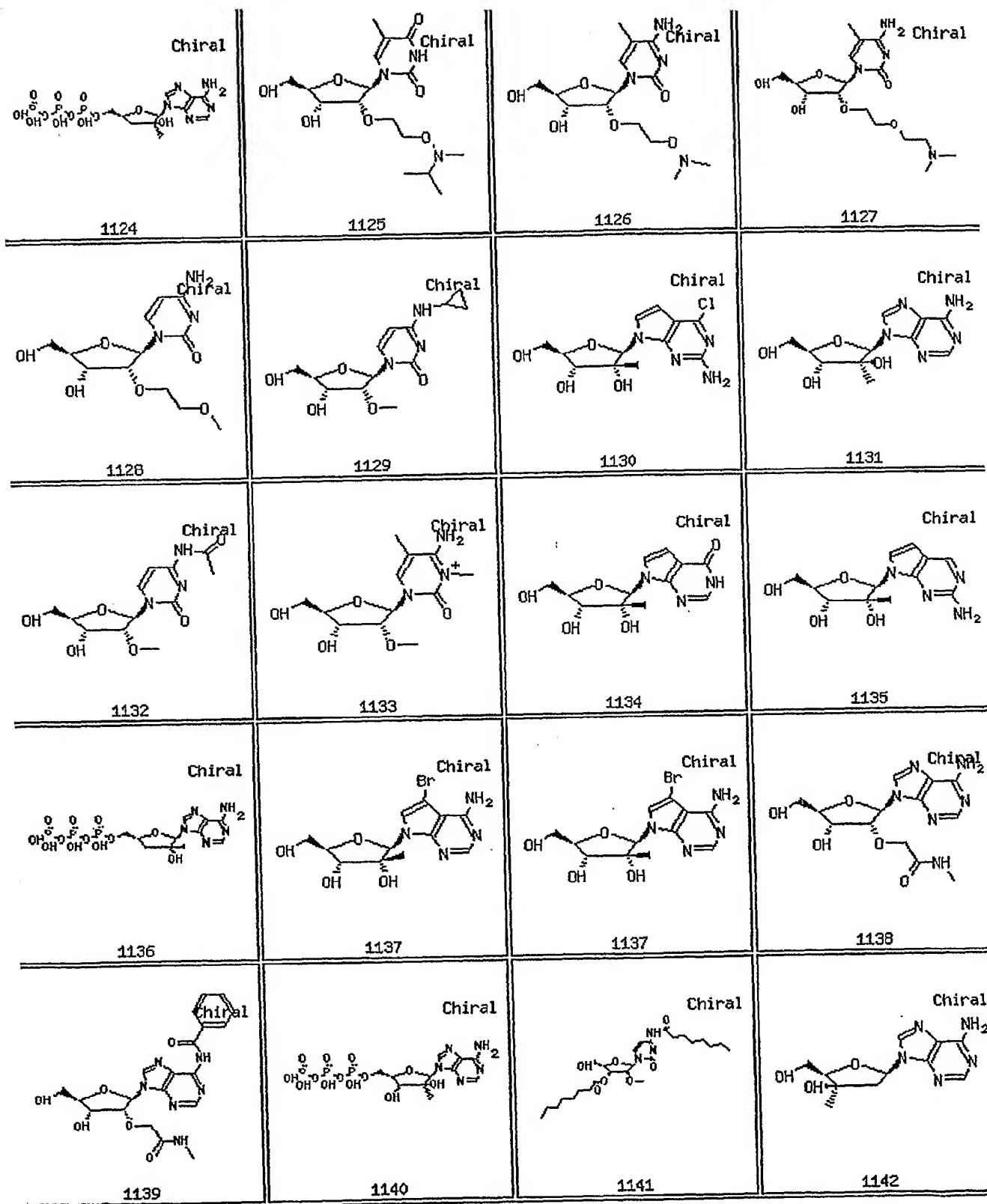


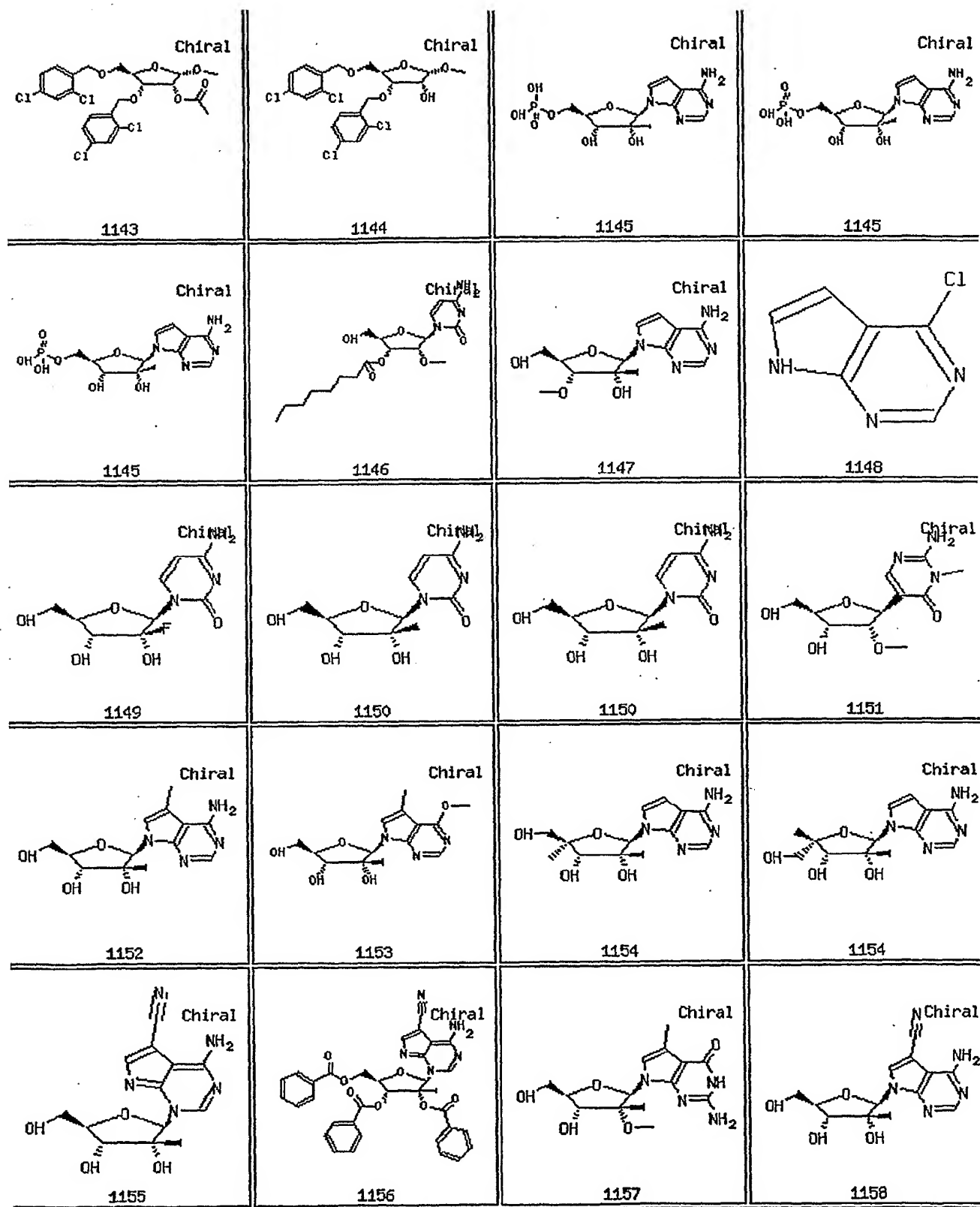
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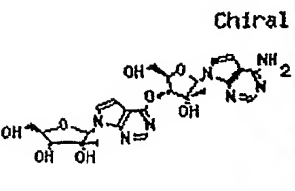
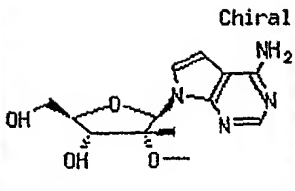
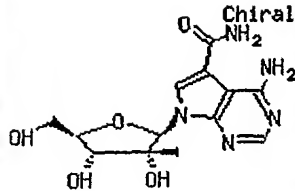
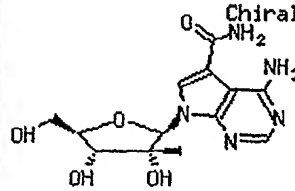
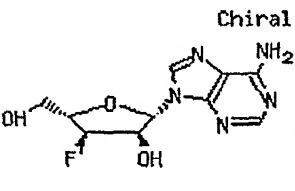
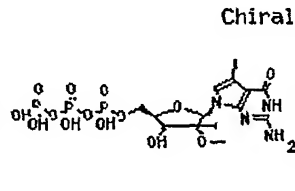
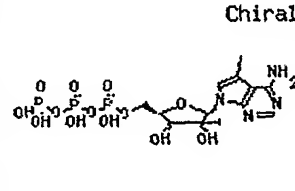
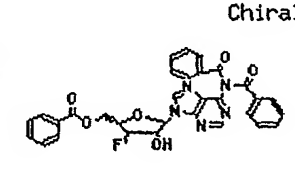
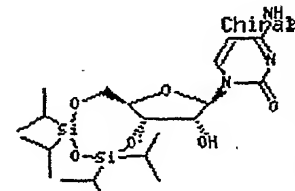
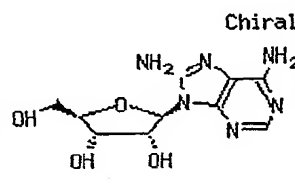
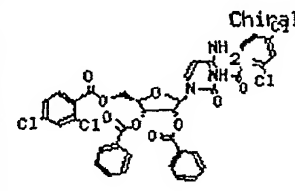
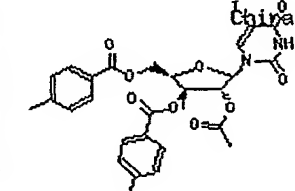
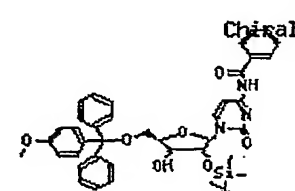
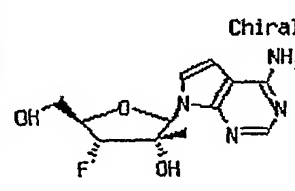
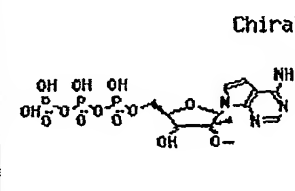
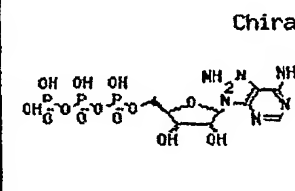
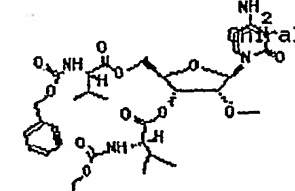
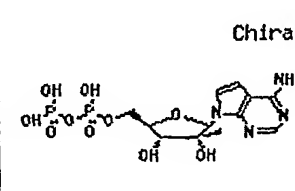
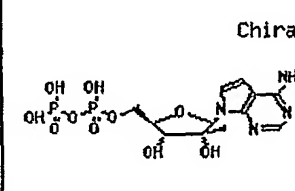
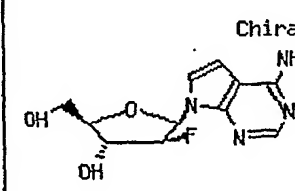
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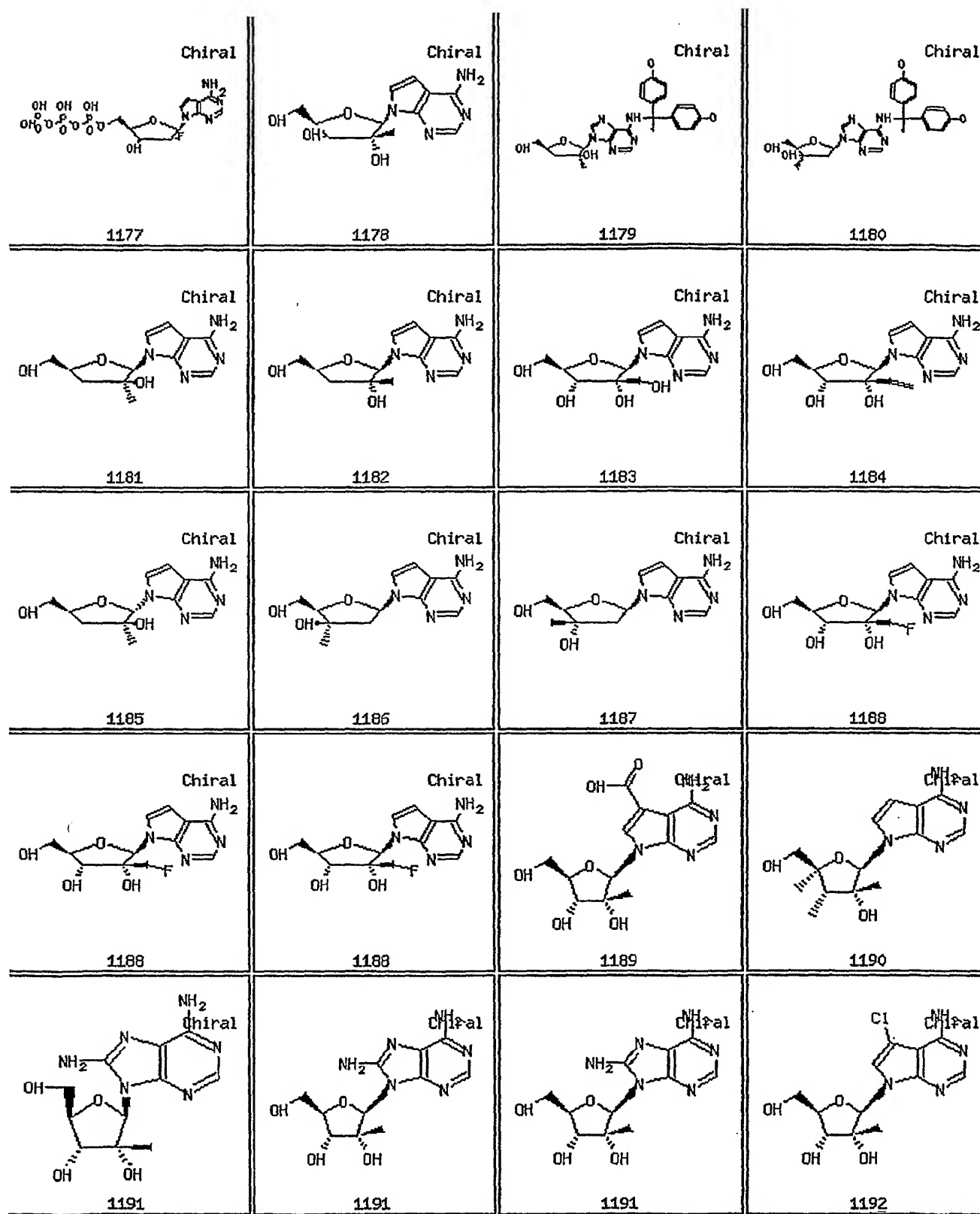
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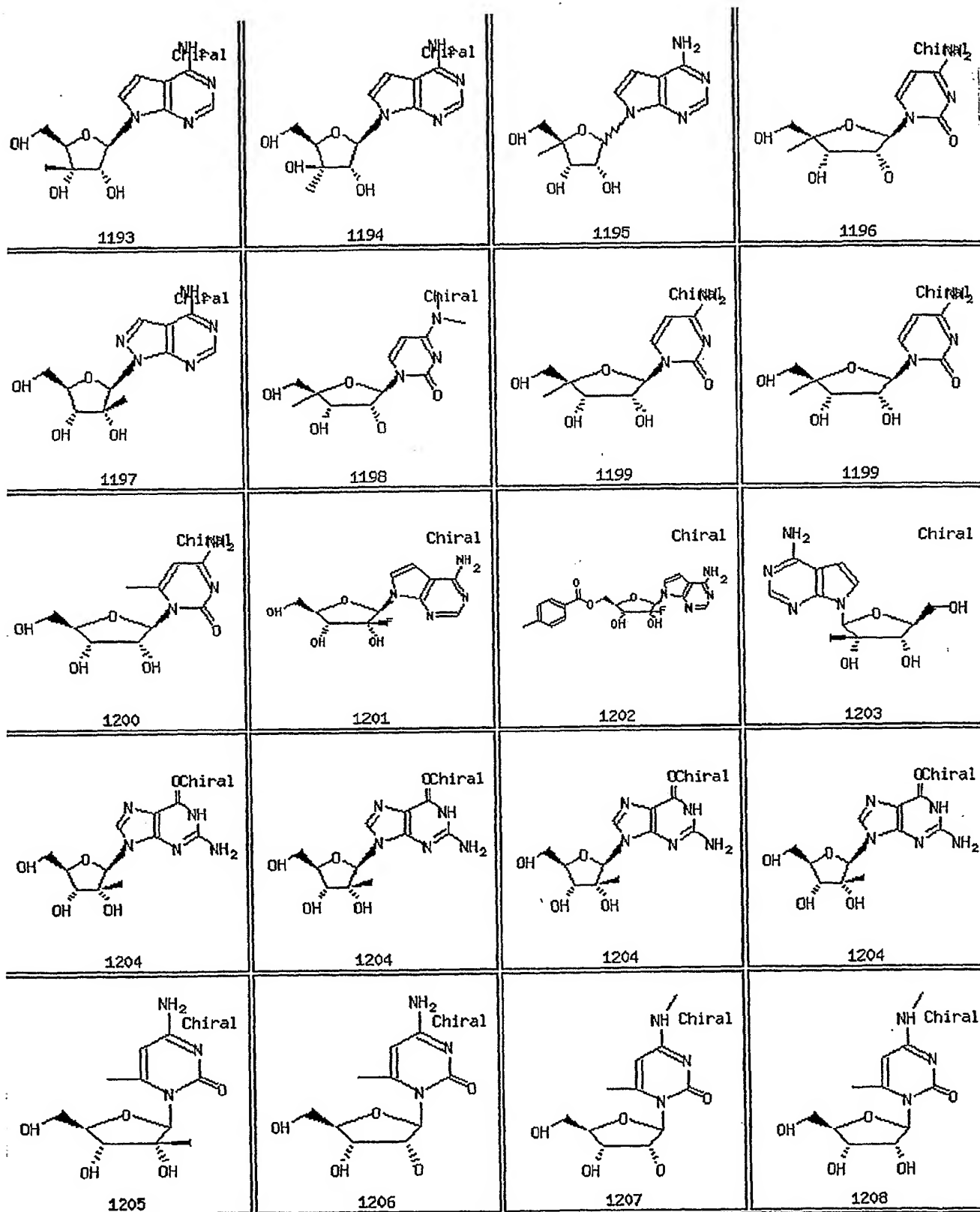


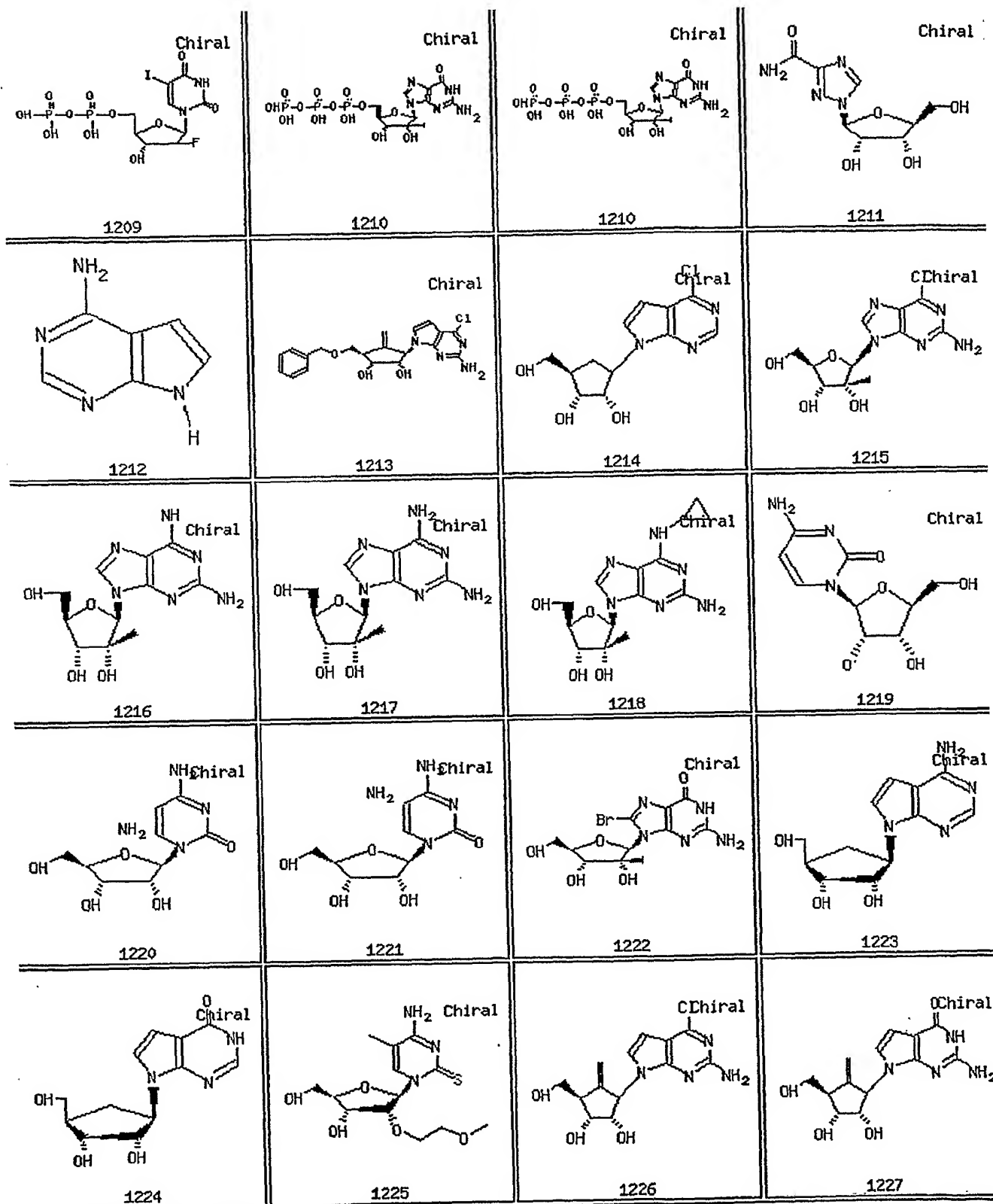


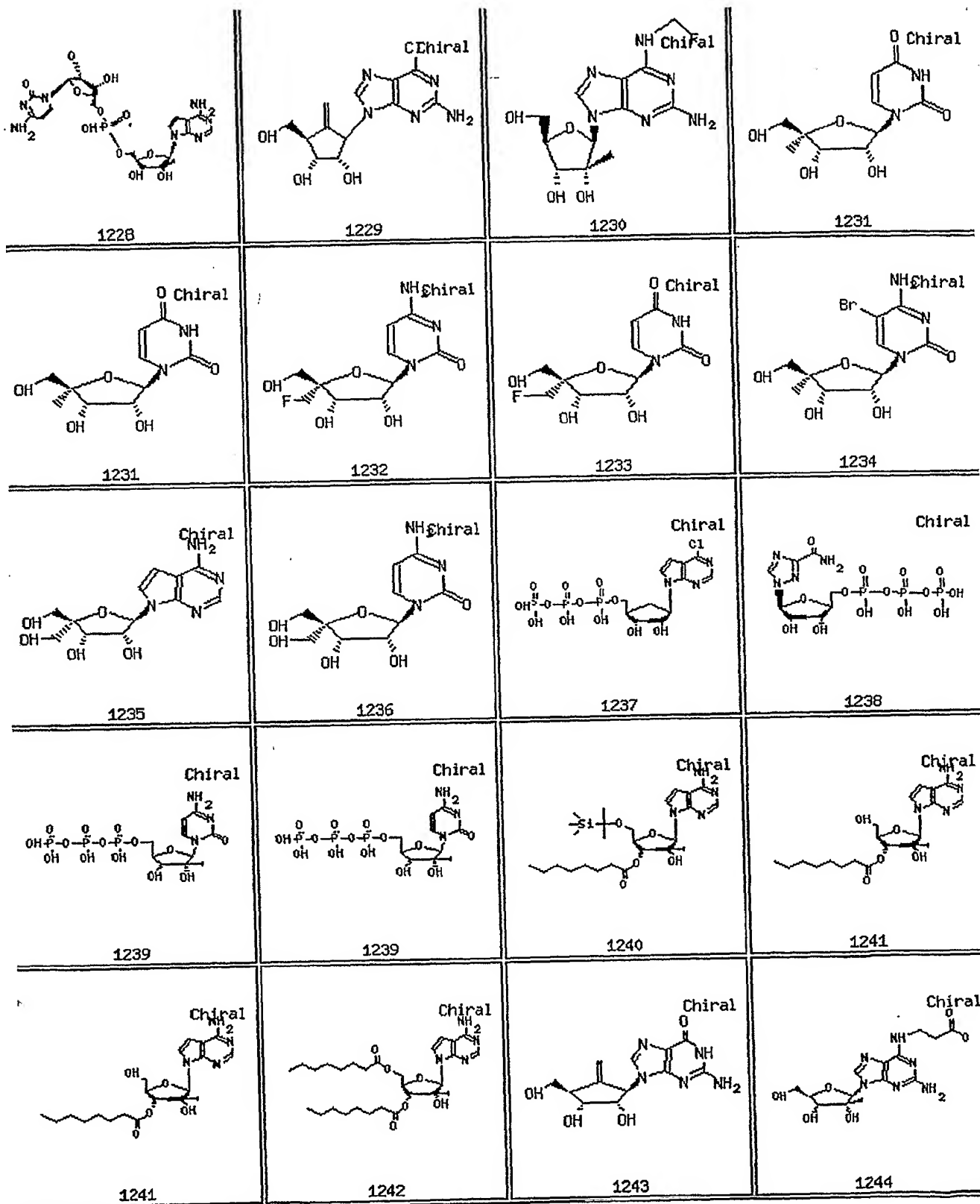
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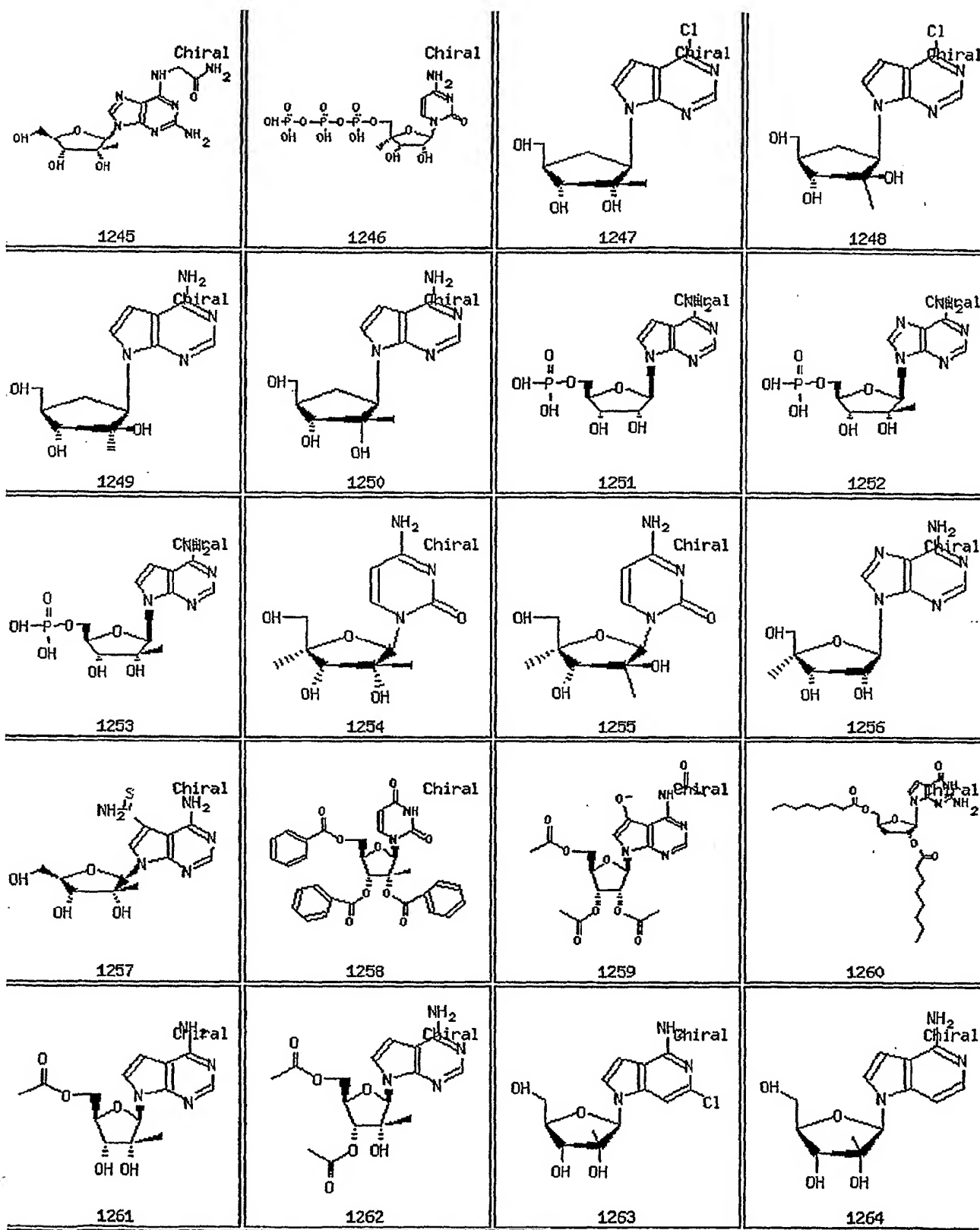


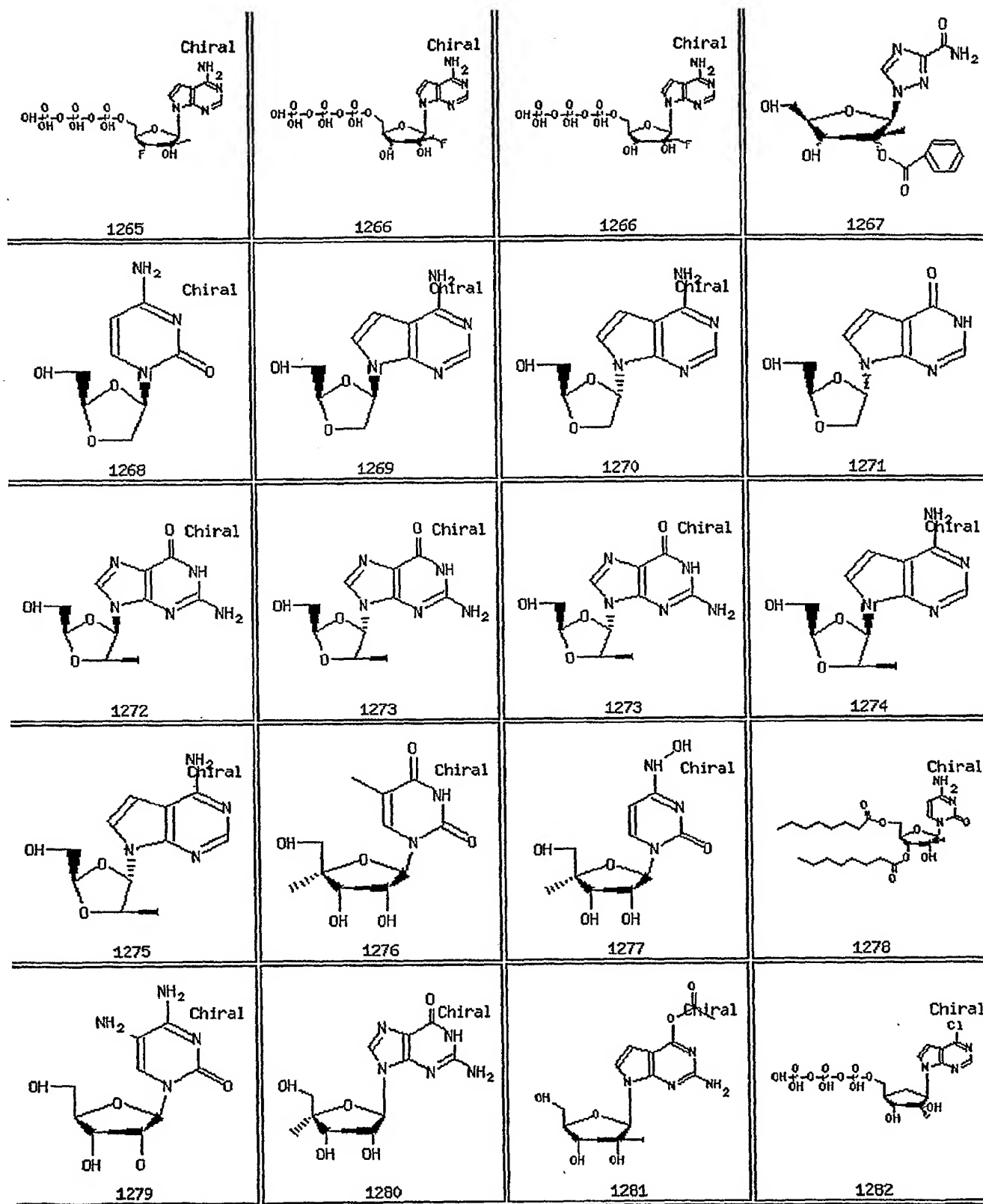


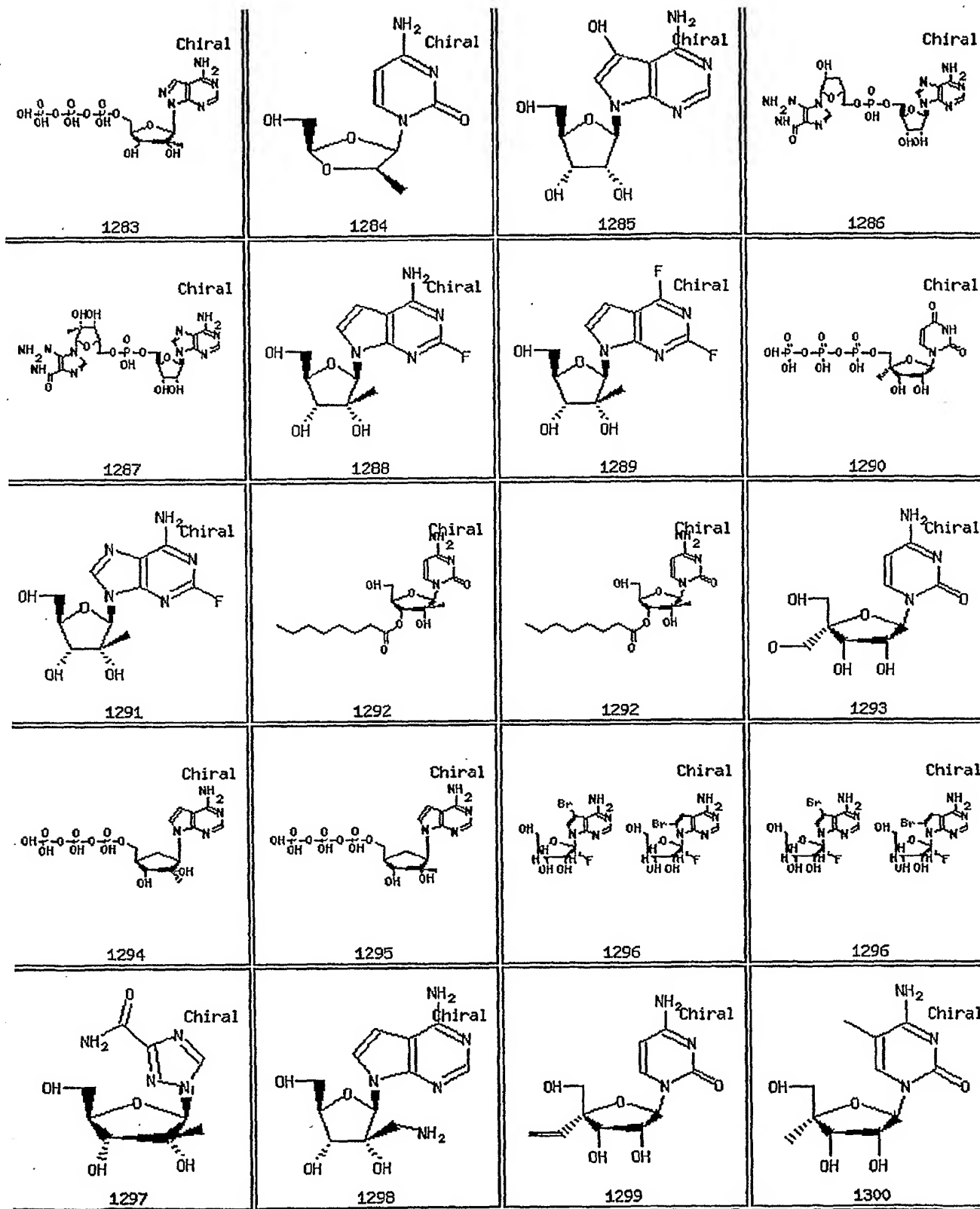


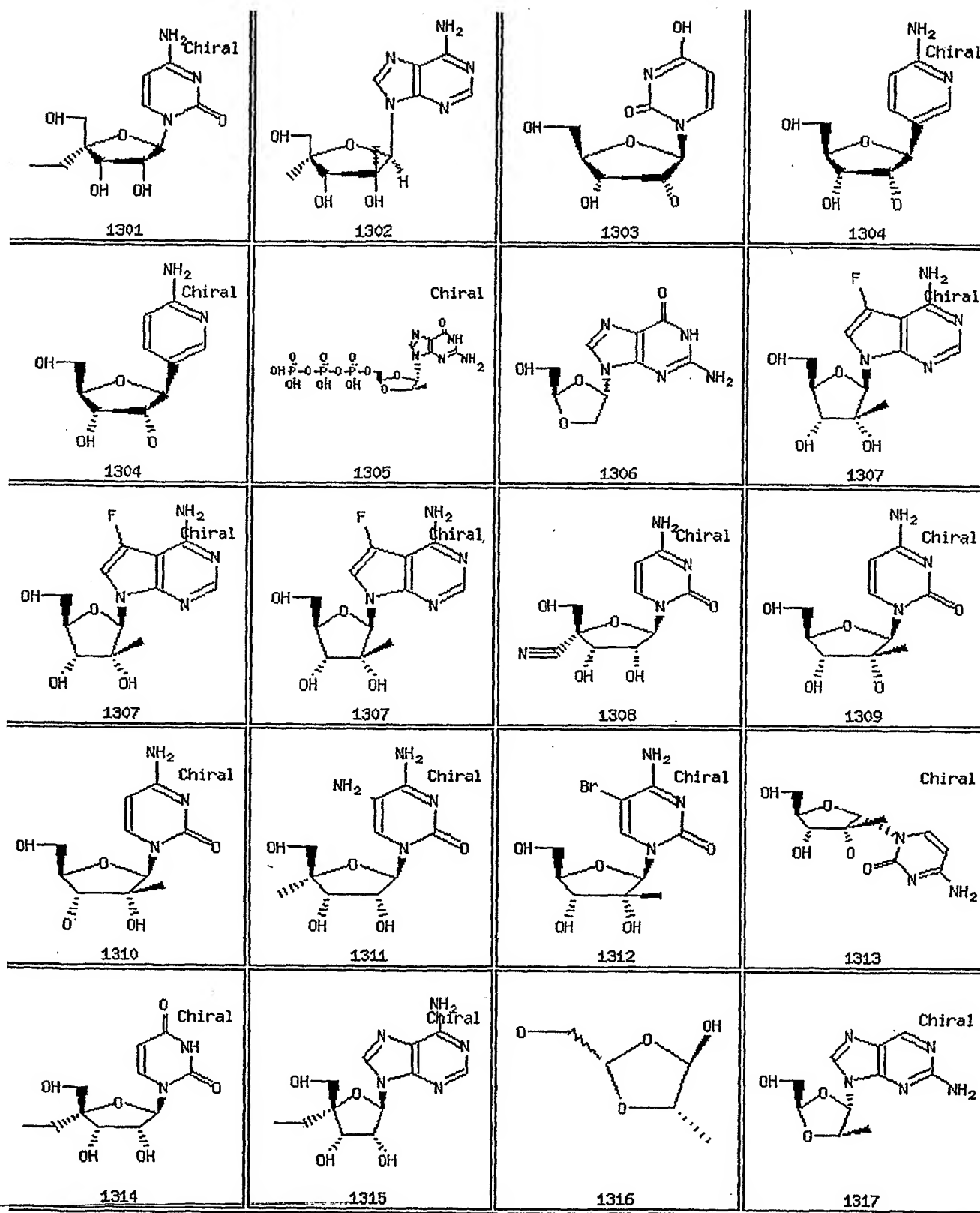




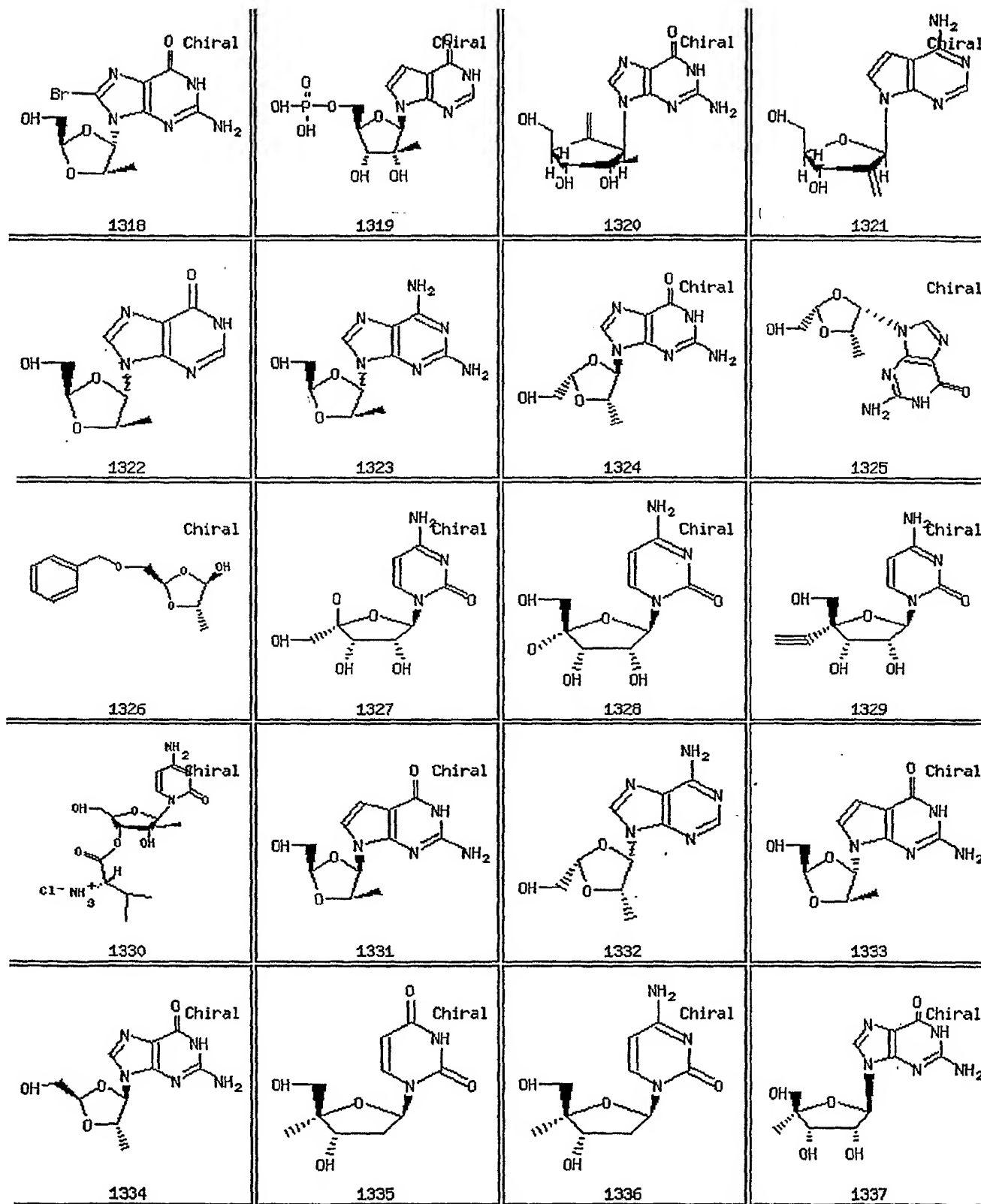


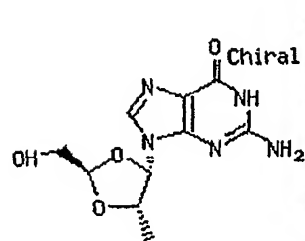




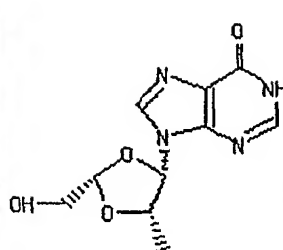




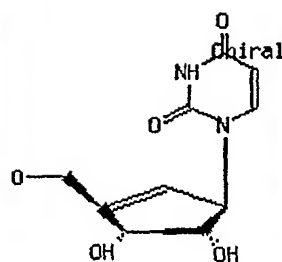




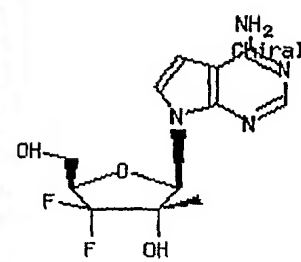
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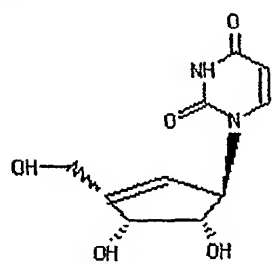
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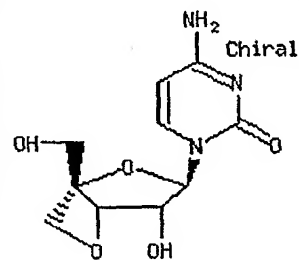
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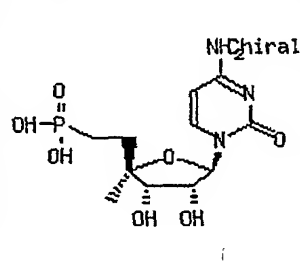
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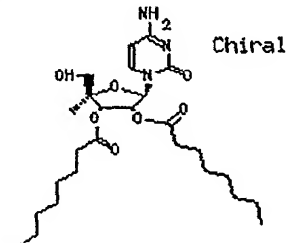
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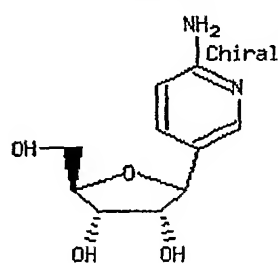
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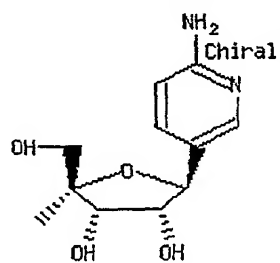
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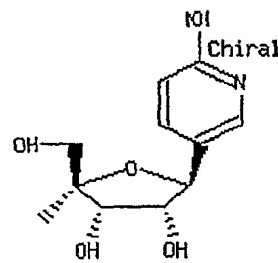
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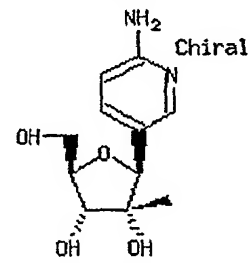
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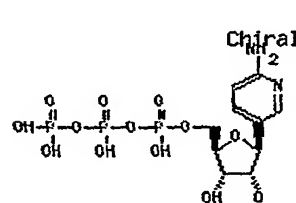
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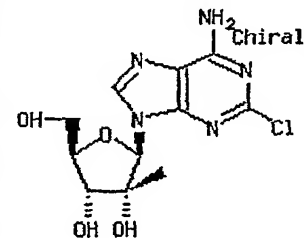
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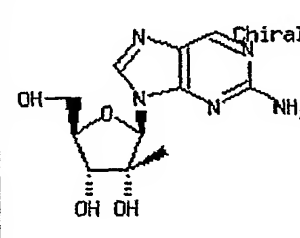
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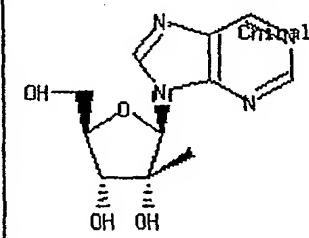
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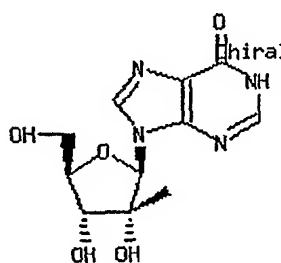
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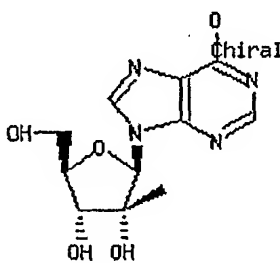
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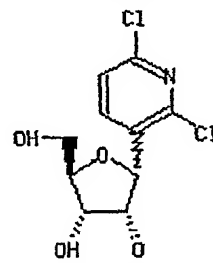
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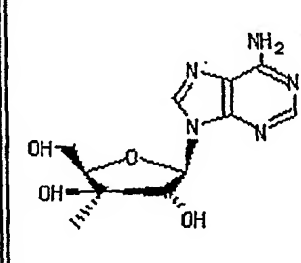
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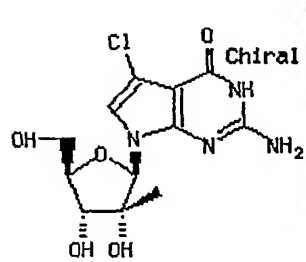
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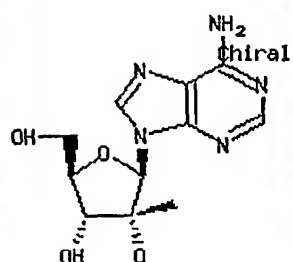
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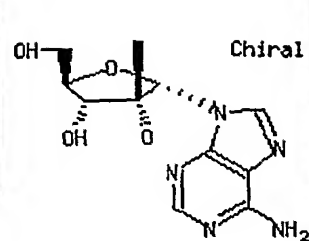
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**WHAT IS CLAIMED IS:**

1. A method of treating a coronavirus infection comprising contacting a mammal having the coronavirus infection with an oligomeric compound, or composition comprising the same, wherein the oligomeric compound comprises at least one antiviral nucleoside and/or nucleoside  
5 mimetic.
2. The method of claim 1 wherein the composition comprising the oligomeric compound is in a form suitable for administration via a pulmonary route.
- 10 3. The method of claim 1 wherein the composition comprising the oligomeric compound is in a form suitable for administration via a nasal route.
4. The method of claim 1 wherein the oligomeric compound is an antisense compound and the composition is in a form suitable for administration via a pulmonary route.  
15
5. The method of claim 1 wherein the oligomeric compound is an antisense compound and the composition is in a form suitable for administration via a nasal route.
6. The method of claim 1 wherein the composition further comprises water.  
20
7. The method of claim 1 wherein the composition is an isotonic saline solution.
8. The method of claim 1 wherein the composition is a buffered saline solution.
- 25 9. The method of claim 1 wherein the composition comprises one or more bioadhesives.
10. The method of claim 1 wherein the composition comprises one or more penetration enhancers.

11. The method of claim 1 wherein the composition comprises a fatty acid penetration enhancer.

12. The method of claim 1 wherein the fatty acid penetration enhancer is sodium laurate or  
5 sodium caprate.

13. The method of claim 1 wherein the composition comprises a liposome.

14. The method of claim 1 wherein the composition comprises a colloidal suspension.

10

15. The method of claim 1 wherein the composition comprises a second antiviral compound.

16. The method of claim 1 wherein the composition comprises an immune stimulatory  
15 agent.

17. The method of claim 1 wherein the composition comprises an emulsifier.

18. The method of claim 1 wherein the composition is a dry powder formulation.

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19. The method of claim 1 wherein the composition is present in a dropper suitable for instilling drops of composition to the nose.

20. The method of claim 1 wherein the composition is present in a device suitable for  
25 delivering single metered doses to the lung.